Second Edition

Microbial Limit and Bioburden Tests

Validation Approaches and Global Requirements

Lucia Clontz

CRC Press
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Preface

In recent years, the field of pharmaceutical microbiology has experienced numerous technological advances, accompanied by the publication of new and harmonized compendial methods. It is therefore imperative for microbiologists who are responsible for monitoring the microbial quality of pharmaceutical/biopharmaceutical products to keep abreast of the latest changes.

Microbial Limit and Bioburden Tests: Validation Approaches and Global Requirements is a reference book for managers, supervisors, and scientists in the pharmaceutical and biopharmaceutical industries engaged in monitoring the microbiological quality of nonsterile pharmaceutical products. This book is also intended for pharmaceutical engineers, quality assurance managers, and other individuals responsible for the microbiological quality of facilities, equipment, and water systems used in production.

In this book, the reader is guided through the various microbiological methods listed in the compendia with easy-to-follow diagrams and approaches to validations of such test methodologies. This new edition is the culmination of months of research and discussions with technical experts, as well as United States Pharmacopeia (USP) and Food and Drug Administration (FDA) representatives, on various topics of interest to the pharmaceutical microbiologist. As such, this new edition presents the most up-to-date information on microbial contamination and control, pharmaceutical waters, environmental monitoring, bioburden testing in support of equipment cleaning validation, quality control testing of microbiological media, and microbiological attributes of pharmaceutical articles. New in this book is an entire chapter dedicated to the topic of biofilms and their impact on pharmaceutical and biopharmaceutical operations. The subject of rapid methods in microbiology has also been expanded and includes a discussion on the validation of alternative microbiological methods and a case study on microbial identification in support of a product contamination investigation.

In the new millennium, both new and continued developments in the field of applied microbiology pose challenges and opportunities to microbiologists who support the production of pharmaceutical and biopharmaceutical products. Among them are the use of alternative microbiological methods, global harmonization, and validation of compendial methods. *Microbial Limit and Bioburden Tests: Validation Approaches and Global Requirements* is a compilation of global regulatory documents and compendial methods, scientific approaches, microbiological concepts and principles that can be used for validation studies and testing in support of pharmaceutical production. Given the complexity and widespread application of microbiology, the author hopes this book will allow pharmaceutical microbiologists to understand the fundamental issues associated with microbiological control and microbial testing and provide them with tools to create effective microbial control and testing programs for the areas under his or her responsibility.

The time spent writing this book was made more pleasurable by the opportunity to discuss with many colleagues in the industry approaches to microbial contamination control and the application of rapid methods to pharmaceutical microbiology.

Finally, I would like to thank my husband, Jim, for his words of encouragement while this book was being written. His companionship has enriched my life and his loving support has given me the strength and determination to always follow my dreams.

Lucia Clontz
June 2008

The Author

Lucia Clontz is director of microbiology at Diosynth Biotechnology, a part of Schering-Plough, located at Research Triangle Park (RTP), North Carolina. Lucia has expertise in pharmaceutical microbiology, and for more than 20 years has managed numerous validation projects, established quality systems for microbiology laboratories, and participated in compliance audits. In her previous position as a consultant and director of regulatory compliance at Serentec, Inc., Lucia helped clients find effective and practical solutions for microbiology issues associated with facilities, processes, and laboratories. Lucia has a B.S. degree in chemistry, with a minor in chemical engineering, and a B.S. degree in marine sciences. She also holds a M.S. degree in microbial biotechnology and a graduate certificate in molecular biotechnology from North Carolina State University. Lucia is a member of the Parenteral Drug Association (PDA), a cofounder of the Pharmaceutical Microbiology Forum (PMF) and the Biofilm Networking Group, and a member of the USP's Council of Experts for Pharmaceutical Waters.

1 Microbial Life and Ecology

The control of microbial contamination requires an understanding of the nature and ecology of microorganisms that might come into contact with the products being manufactured. In this chapter, an overview of microbiology with an emphasis on bacteriology (the study of bacteria), mycology (the study of fungi), and the compendial test organisms will be presented.

AN OVERVIEW OF MICROBIAL LIFE

A microorganism or microbe can be defined as a living organism so small that it can only be observed through a microscope. Microorganisms consist of single cells or clusters of cells. A microbial cell is the fundamental unit of life; it maintains the structure of the microbe by taking up chemicals and energy from the environment and by responding to stimuli from its surroundings. Microbes reproduce and pass on their genetic makeup to their offspring, and evolve and adapt to the environment. Single-celled microorganisms were the first life form to appear on earth approximately 3.5 billion years ago [1] and over the past 1.5 billion years there has been a tremendous diversification of life, culminating in complex multicellular organisms such as plants and animals. In many cases, microorganisms have evolved to live in intimate symbiotic relationships with other organisms, including plants and animals. Human beings, for example, have mutualistic relationships with many bacteria that live in the human gut. In other extreme cases, through evolution, microbes have been assimilated by and become part of other cells; many recent studies have provided hard evidence that chloroplasts and mitochondria were once free-living bacteria that were incorporated into eukaryotic cells to become energy-producing organelles [1].

Microorganisms are ubiquitous, and their natural habitats are extremely diverse. They survive within a wide range of temperature, pH, salt concentration, nutrients, available water, and other environmental factors. Microbes have been found in hot springs, on deep sea hydrothermal vents, in ice, in deserts, deep inside rocks, and even under extreme harsh chemical conditions where life was once believed not to be possible; for example, microbes have been found in rocks 7 km below the surface of the earth, and others survive in vacuum and may even be able to survive in space. These organisms are referred to as *extremophiles*, that is, organisms that grow optimally and thrive under extreme chemical or physical conditions (e.g., pH <1, pH >12, 113°C, high salinity, etc.) [2].

Biodiversity is indeed the beauty of microbiology: the ability of microorganisms to survive and thrive in almost any condition. However, to pharmaceutical companies, this gift of life is extremely detrimental and poses great risks to the quality and safety of drug products because, in most cases, carrying out manufacturing operations under complete sterile conditions is nearly impossible. Therefore, the control of microbial contamination of pharmaceutical processes is a challenging task that must be managed by skilled and knowledgeable scientists.

Microbial Phylogeny

The evolutionary relationship between microorganisms is called *phylogeny*. With the recent advances in molecular biology and the use of comparative gene sequencing of 16S or 18S ribosomal(r) ribonucleic acid (RNA), three phylogenetically distinct lineages of cells have been identified: the Bacteria, the Archaea, and the Eukarya (see Figure 1.1). These three evolutionary domains replaced the five-kingdom system that overemphasized the evolutionary importance of multicellular plants and animals, whereas the reality is that most evolutionary diversity actually resides within the world of microorganisms.

The Bacteria and Archaea domains contain only prokaryotes, which are simple cells that lack a nucleus and other membrane-enclosed organelles. Eukaryotes, organisms from the domain Eukarya, have a membrane-bound nucleus (true nucleus) and other organelles. All three domains are believed to have originated from a common ancestral organism, often referred to as the "universal ancestor." One interesting

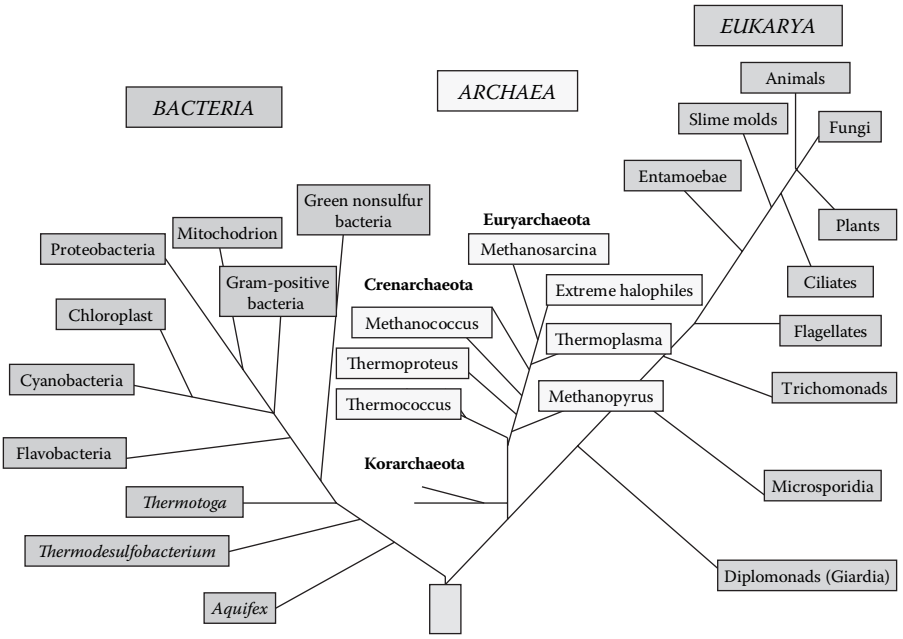


FIGURE 1.1 Universal phylogenetic tree. (Adapted from *Brock Biology of Microorganisms* (2003), 10th ed., Pearson Education.)

fact is that organisms from the domain Archaea are actually more closely related to *Eukarya* than *Bacteria*.

A fourth group of biological entities also studied in the field of microbiology is the *virus,* which is not an organism in the same sense that eukaryotes, archaeans, and bacteria are. Viruses are genetic elements comprised of a piece of nucleic acid (either RNA or deoxyribonucleic acid [DNA]); a *virion* is the complete virus particle whose nucleic acid is surrounded by a protective protein coat. There are also other entities that normally would not be considered viruses but which are viruslike in their behavior: the two most important ones are viroids and prions. *Viroids* are the smallest known pathogens with only a single strand of RNA molecule; *prions* do not seem to have any nucleic acid, but have a distinct extracellular protein coat. Viruses, viroids, and prions are not considered living organisms, because they are totally dependent on host cells that they infect for their metabolism and reproduction. Certain viruses can insert their genetic material into a host's genome and literally take over its biological functions. Although viruses, viroids, and prions have significant biological importance, especially to biopharmaceutical manufacturing, this topic is outside the scope of a compendial bioburden and microbial limit testing program. However, the pharmaceutical microbiologist should become familiar with virology (the study of viruses) principles, as well as viral clearance and viral testing programs. The microbial quality of cell lines and culture media used in fermentation (e.g., bacterial and fungal) and cell culture (e.g., mammalian and insect) processes used in production of recombinant proteins is often the responsibility of the quality control (QC) microbiologist. In addition, the QC microbiologist may also be involved in the design of facility- and equipment-cleaning programs that are capable of viral inactivation/viral clearance.

Microbial Taxonomy

Taxonomy is defined as the scientific classification and nomenclature of organisms. On the basis of genotypic analysis, microorganisms are grouped into seven categories: domain, phylum, class, order, family, genus, and species. This type of classification is useful because it allows microbiologists to establish relationships and possible common sources of contamination among microbial isolates. For example, the taxonomy for the classic microorganism *Escherichia coli* is as follows (note that the genus and species of a microorganism are always represented in italics):

- Domain: Bacteria
- Phylum: Proteobacteria
- Class: Gamma-proteobacteria
- Order: Enterobacteriales
- Family: Enterobacteriaceae
- Genus: *Escherichia*
- Species: *coli*

Fungi are classified based on their life cycle patterns, and over the years taxonomy of fungal isolates has been much of a challenge because different names have been

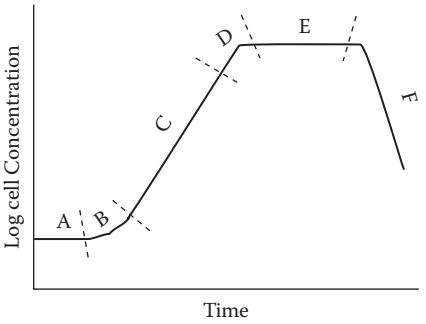
assigned for the same type of organism. Nomenclature of filamentous fungi is part of a classification system based on the organism's appearance, especially their sexual stages, rather than on biochemical reactions and nutritional requirements that are key to classification of bacteria. Therefore, the same mold may have two or more names: one for the sexual state (teliomorph) and one or more describing the asexual conidia-producing forms (anamorph). With the advances in molecular biology techniques, nucleic acid sequencing may provide more objective and a more natural taxonomic method for separation of the various genera and species of fungal isolates, thus making contamination source identification and trending an easier task for the pharmaceutical microbiologist. For more information on taxonomy of microorganisms, the author recommends the *Bergey's Manual of Systematic Bacteriology* [3] and the article "Developments in Fungal Taxonomy" by Guarro et al. [4].

Microbial Growth and Survival

Microorganisms have diverse growth requirements, but they are also capable of quickly adapting to different environmental conditions by turning on and turning off genes. Through a process called *metabolism*, microorganisms use nutrients available to survive and reproduce. Metabolism can be divided into two processes: (1) *anabolism,* in which cells use energy to create complex molecules such as cell structures, and (2) *catabolism,* in which cells break down complex molecules to generate and store energy. Microbes have basic needs to be able to undergo metabolic processes, and those include energy sources and chemical sources (nutrients) for their cellular structures. In addition, metabolism is highly dependent on factors such as temperature, pH, water, and oxygen availability. These are key growth conditions that must be balanced in an enrichment medium and during incubation to allow microbes to metabolize, grow, and reproduce and therefore be detected by traditional microbiological methods.

Growth Curve

The usual growth kinetics of microorganisms involves an initial phase called the *lag phase*, in which no apparent growth occurs. It is a period of adaptation to the nutrient medium/environment. In the following phase, called the *exponential phase*, the organisms multiply very fast at a constant rate. This growth phase is characterized by a doubling time (generation time) and a net increase in cell population. During the log phase, cells produce primary products of metabolism that are essential for their growth (e.g., amino acids, lipids, proteins, etc.). Following this rapid-growth phase, microbial growth starts to slow down because of depletion of critical nutrients (substrate limitation) and accumulation of toxic products, and the organisms reach a *stationary phase*. During this phase, many organisms produce secondary metabolites such as enzymes or antibiotics to improve their chance of survival, and the microbial population remains stable over time until the organisms enter the decline phase called the *death phase*. During this last phase, cells are unable to regenerate, thus leading to a net decrease in cell population as a result of cell lysis and death. Figure 1.2 is a representation of the typical microbial batch culture growth kinetics. However, in nature, most microbes do not follow this type of growth pattern, because they rarely encounter the optimum growth conditions seen in a microbiol-



Section of Curve	Phase	Growth Rate
A	Lag	Zero
B	Acceleration	Increasing
C	Exponential	Constant
D	Retardation	Decreasing
E	Maximum stationary	Zero
F	Decline	Negative (death)

FIGURE 1.2 Phases of microbial growth.

ogy laboratory setting or in bioreactors. In fact, most environmental isolates live under stressed conditions and in communities (biofilms) that help them adapt to their surroundings and survive the environmental stresses. This topic will be addressed in detail in Chapter 10.

Temperature

Depending on temperature ranges that provide for optimum growth conditions, microorganisms are classified into four groups (see Figure 1.3). These are referred to as *cardinal temperatures*, that is, the minimum, maximum, and optimum growth temperatures for the various types of organisms:

- *Psychrophiles*: Organisms with low temperature optima of 15°C or lower (approximate range: 0°C to <20°C). Note: Organisms that are able to grow at 0°C but have temperature optima in the range of 20–40°C are classified as *psychrotolerant*.
- *Mesophiles*: Organisms with temperature optima of 37°C (approximate range: 20–45°C).
- *Thermophiles*: Organisms with temperature optima of 60°C (approximate range: 45–80°C).
- *Hyperthermophiles*: Organisms with temperature optima of 80°C or greater.

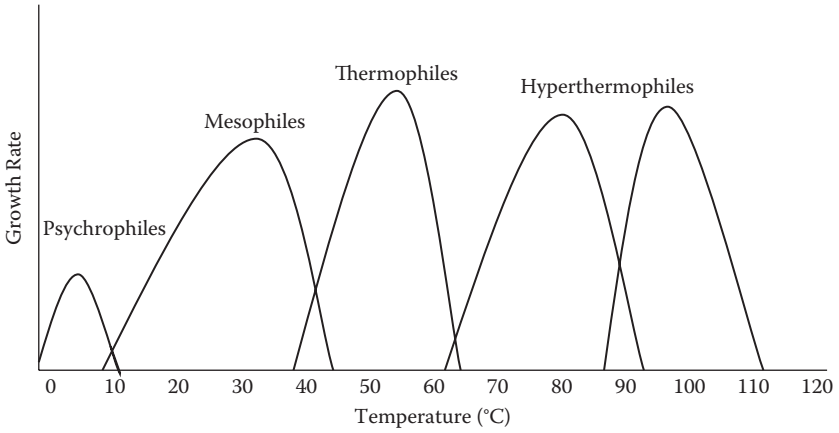


FIGURE 1.3 Temperature classes of microbes and relation to growth rate.

Many of the prokaryotes that grow in the most extreme environments are from the domain Archaea, although some bacterial and fungal extremophiles also exist. Thermophiles and hyperthermophiles offer many advantages to biotechnological processes because their proteins/enzymes are very heat stable. One example that is familiar to the molecular biologist is the *Taq polymerase,* an enzyme from the thermophile *Thermus aquaticus* that is widely used in polymerase chain reaction (PCR) procedures.

Although microbes are classified based on their preferred temperature range for growth, these temperature ranges can and do vary depending on the growth medium, and variances are even seen among species of the same organism. It also seems that the study of optimum growth conditions of microbes is an area in microbiology that is ever changing as viable microbes continue to be discovered in places under even more extreme physical and chemical conditions.

Energy Sources

All living organisms require energy for survival, and all cells need carbon as a major nutrient. Energy can be obtained from organic chemicals, inorganic chemicals, or light. Energy is obtained by the oxidation of chemical compounds and conserved in the cells as adenosine triphosphate (ATP). The manner in which organisms utilize available resources for survival will determine their metabolism. As such, microorganisms can be classified as follows:

- *Heterotroph*: Microbes that require one or more organic compound as carbon source
- *Autotroph*: Microbes that use CO_2 solely as the carbon source
- *Chemolithotroph* (many are autotrophs): Organisms that obtain energy from the oxidation of inorganic compounds
- *Chemoorganotroph* (heterotroph): Organisms that obtain energy from the oxidation of organic compounds
- *Phototroph*: Organisms that obtain energy from light

Oxygen

The chemical reactions (metabolism) that are carried out by microorganisms to obtain, store, and use energy may or may not require oxygen. Depending on the need for oxygen, microorganisms are classified as follows:

- *Aerobes*: These are organisms that need oxygen for their metabolism.
- *Strict anaerobes*: These are organisms that either cannot grow or must grow in the absence of oxygen. For example, anaerobic bacteria obtain energy for their metabolism through fermentation (a process of breaking down organic molecules with enzymes).
- *Facultative anaerobes*: These microbes are able to grow in the presence of oxygen.

In order for the microbiologist to recover viable microorganisms from the environment or from a sample, the proper nutrient medium and incubation conditions must be used so that microbes will be able to grow and be detected. Compendial methods for bioburden and microbial limit testing use media designed for the recovery of heterotrophic mesophilic aerobic or facultative anaerobic organisms, because those are the most common contaminants of pharmaceutical facilities and products. For example, trypticase (tryptic) soy agar (TSA) is a typical all-purpose medium designed for the recovery of a broad spectrum of environmental bacteria and fungi. However, in some cases, a defined/minimal medium or alternate incubation conditions may be needed to grow specific organisms of concern. As we shall see later in this chapter, other types of media are used to either select for specific organisms or to differentiate, based on their colonial morphology, certain organisms from others that are growing on the same medium.

In terms of types of microbial contaminants, most microorganisms associated with contamination of pharmaceutical products are bacteria from the phyla Proteobacteria and Gram-positive bacteria, and eukaryotic fungi (yeasts and molds). These organisms are typical isolates from pharmaceutical environments, and many are known human pathogens or opportunistic pathogens. Therefore, in this chapter we will focus on these specified types of organisms.

BACTERIA

The domain Bacteria contains a great variety of prokaryotes, including all known pathogenic bacteria and many opportunistic human pathogens. Bacteria are ubiquitous single-celled prokaryotes that are very adaptable to all types of environmental conditions. Bacteria can be found everywhere, from the almost airless layers of the atmosphere to attached populations (biofilms) in frozen soil and rocks in hot springs. Bacteria are very small: an average rod-shaped bacterium such as *E. coli* is generally between 1–3 μm in length and about 1 μm wide [1]. However, size varies based on the metabolic state of the organism, and many bacteria, for example, the putative nanobacteria, can be as small as 0.1–0.2 μm in diameter [1]. For manufacturers of sterile drug products that rely on filtration processes that use 0.1–0.2 μm pore size

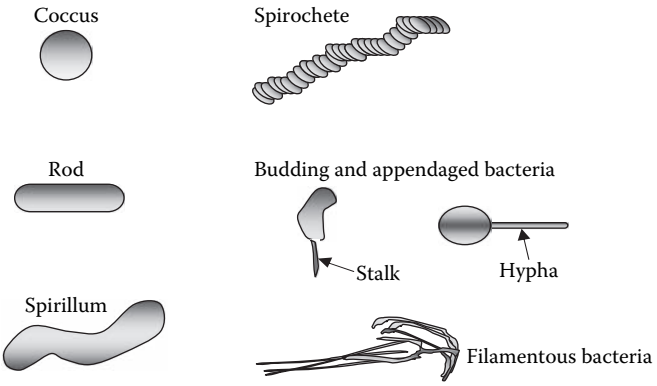


FIGURE 1.4 Different types of bacterial cell shape.

membrane filters, the possibility of having nanobacteria/ultramicrobacteria in their products is a serious and valid concern.

Cell Shape

Most bacteria have a rigid cell wall that gives them characteristic shapes when observed under a microscope and allows them to be classified as follows (see Figure 1.4):

- Rod: A bacterium with a cylindrical shape.
- Coccus (plural: cocci): A bacterium having a spherical or ovoid shape. When the cells form chains, they are called streptococci; if they are in clumps, they are called staphylococci.
- Spirillum (plural: spirilla): A bacterium in the shape of a rod that is curved and forms spiral-shaped patterns.
- Spirochete: A bacterium having a tightly coiled shape.
- Budding and appendaged: Bacteria that have extensions of their cells as long tubes or stalks.
- Filamentous: Bacteria that form long thin cells or chains of cells.

The cell shape characteristics of certain types of bacteria may change under given growth conditions, and this is an important fact that must be taken into account when performing microbial identifications using traditional methods (Gram staining and microscopic examinations). Many microbiologists also attempt to identify bacteria on the basis of their colonial morphology on solid media. Although a useful preliminary tool for the trained microbiologist, the size, shape, texture, and color of microorganisms may and do change significantly depending on growth conditions and type of nutrient media used.

Mycoplasma

Mycoplasmas are the smallest of bacteria (0.2–0.3 μm) with the minimum amount of DNA needed to code for a functioning cell. Mycoplasmas also lack a cell wall,

which other bacteria have, and are therefore shapeless and highly pleomorphic. Unlike other bacteria, on solid media, mycoplasmas have a characteristic "fried egg" appearance. Most mycoplasma organisms are intracellular or animal parasites because they need protection from environmental stresses, given the fact that they lack a cell wall. Mycoplasmas also require sterols to provide stability for their cytoplasmic membrane. As such, mycoplasma contamination of traditional pharmaceutical manufacturing products and facilities is not a concern; however, these organisms are of great concern to biopharmaceutical manufacturing because mycoplasmas can be potential contaminants of cell lines and cell culture media. The fact that mycoplasmas are so small and can take up any shape is also a challenge to filter manufacturers that develop filtration devices for the purpose of rending cell culture media and other materials sterile and free of mycoplasma contamination. Mycoplasmas are also of great medical importance because of their human pathogenicity and resistance to antibiotics, such as penicillin, that inhibit cell wall synthesis. Testing for mycoplasma is not part of a compendial bioburden or microbial limit testing program (e.g., USP Chapters <61> and <62>), and therefore this topic will not be addressed in this book. For those interested in the topic, testing for mycoplasma in support of biotechnology processing is addressed in the USP in the following chapters:

- <1043>, Ancillary Materials for Cell, Gene, and Tissue Engineered Products
- <1045>, Biotechnology-Derived Articles
- <1046>, Cell and Gene Therapy Products
- <1050>, Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin
- <1211> Sterilization and Sterility Assurance of Compendial Articles

Bacterial Growth and Reproduction

Prokaryotes, unlike many eukaryotes, do not undergo sexual reproduction. Bacteria reproduce by binary fission (one cell divides into two, two into four, four into eight, etc.). This growth pattern is referred to as the organism's generation time (doubling time), and it varies greatly depending on the type of microorganism and growth conditions. However, there are certain mechanisms used by bacteria for the transfer of genetic material. For example, certain types of bacteria, such as *E. coli*, can undergo a process called *conjugation*, the transfer of genes from one cell to another involving cell-to-cell contact and a plasmid. Bacteria can also exchange chromosomal material through *transduction,* a process that involves the accidental transfer of bacterial genes between two cells by a bacteriophage (virus) and the incorporation of these genes into the genome of the recipient bacterium.

Most bacteria live as vegetative cells, and therefore, they require moisture and organic matter for survival and proliferation. However, some bacteria when faced with environmental stresses are capable of producing spores that remain in a dormant state for long periods of time; once conditions again become optimum for growth, the spores germinate and create new vegetative cells.

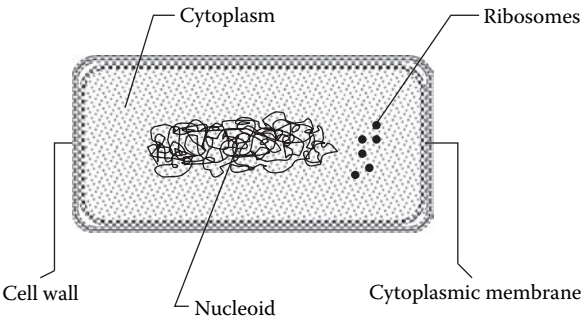


FIGURE 1.5 Diagram of a prokaryotic cell.

Cell Structures

The bacterial cell may appear to be a simple biological structure, but in reality, it is a well-developed biological unit capable of performing unique functions such as *motility* and transduction; in fact, many biological characteristics of Bacteria are not present in organisms from the domains Archaea or Eukarya.

A bacterial cell is basically a contained biological system that has an envelope (membrane) that protects an internal medium (cytoplasm) containing vital biological structures (see Figure 1.5). The intracellular structures of prokaryotes that are contained in the cytoplasm are simple: bacteria do not have organelles as eukaryotes do. As discussed earlier, bacteria lack a true nucleus, and their genetic material (DNA) is present in the cell in a large double-stranded molecule (bacterial chromosome) as a mass referred to as the *nucleoid*. Another key intracellular structure of bacteria is the ribosome—these structures are usually present in large numbers in the cell and are the sites for protein synthesis. Most prokaryotes also contain *plasmids* in their cytoplasm. These are small amounts of circular extrachromosomal DNA that contain genes, which are nonessential for cell growth but that often confer special properties and selective advantages to the organism (e.g., resistance to a particular antibiotic). Plasmids replicate independently of the chromosomal DNA and have proved extremely useful in genetic engineering, being used as vector expression systems for production of recombinant proteins. For example, the bacterium *E. coli* is a model organism for expression of heterologous proteins, using plasmid vectors that incorporate the gene for the protein of interest.

Surrounding the cytoplasm is the bacterial cytoplasmic membrane, a phospholipid bilayer that acts as a permeable barrier for nutrients to enter the cell and for waste to leave the cell. These membranes are also sites for energy conservation where the proton motive force is generated. Located just outside the cytoplasmic membrane is the bacterial cell wall, a porous structure containing peptidoglycan, which is responsible for the shape and integrity of the bacterial cell. Virtually all bacteria have cell walls with peptidoglycan; exceptions include the Planctomyces-Pirella group, which have cell walls composed of protein, and the Mycoplasma-Chlamydia group already discussed earlier in this chapter. In fact, the presence of peptidoglycan in a microbial cell wall is an identifying feature for organisms from the domain Bacteria because both Archaea and Eukarya lack this chemical compound.

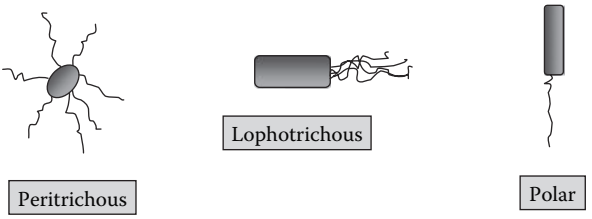


FIGURE 1.6 Flagellar arrangements of prokaryotes.

Many bacteria are motile, a function that is typically associated with a cell structure called the *flagellum* (plural: flagella; see Figure 1.6). Flagella are whip-like structures that protrude from the cell wall and depending on their location and arrangement, they are identified as

- Peritrichous—found at several locations around the cell
- Lophotrichous—tuftlike formation located at one end of the cell
- Polar—a single flagellum found at either end of the cell

Motility is indeed a crucial advantage to a bacterium; the ability to move around confers a selective advantage and serves as a survival tool. For example, through chemotaxis, bacteria can move toward a favorable chemical gradient or away from a harmful one; likewise, through phototaxis, photosynthetic bacteria can move toward light and use it as a needed energy source. Some marine organisms also have gas vesicles that allow them to adjust the gas content inside their cells so that they can move up or down in the aqueous medium toward a favorable environment.

Many bacteria have structures called fimbriae and pili; these are similar to flagella but are not involved in motility and are typically found in pathogenic bacteria. Fimbriae (singular: fimbria) are short, cylindrical protein structures found in high numbers surrounding the bacterial cell surface (see Figure 1.7). Their main function is surface cell adhesion (biofilm formation and attachment to other organisms to initiate infection) and pellicle formation. Contrary to fimbriae, pili (singular: pilus) are long protein tubular structures present on bacterial cells but in low numbers. Pili are thought to be involved in conjugation and therefore are often referred to as *sex pili* (see Figure 1.7). Some pathogenic bacteria use a pilus as a device for surface attachment for infectious purposes (nonsex pilus).

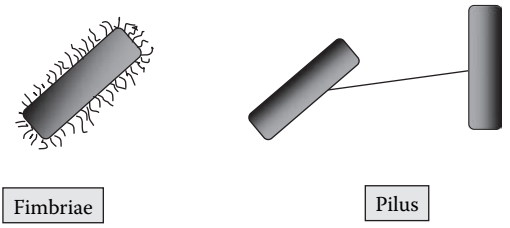


FIGURE 1.7 Bacterial frimbriae and pilus.

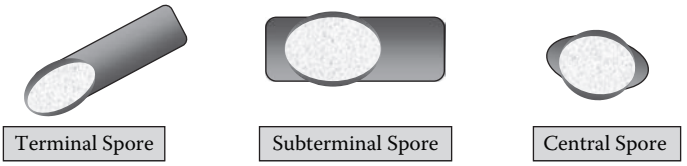


FIGURE 1.8 Types of bacterial endospores.

As discussed earlier, some prokaryotes, unlike other organisms, are capable of forming spores as a means of surviving adverse environmental conditions (see Figure 1.8). These structures confer on certain bacteria high resistance to extreme chemical and physical stresses that otherwise would be lethal to their vegetative cells. As such, the main function of bacterial endospores is survival, and these structures are not part of the reproductive cycle of bacteria—during sporulation only one spore is formed per cell and upon germination, only one cell is created. The location of the endospore within the bacterial cell is species specific and can be used as a microbial identification tool. Endospore formation is limited to Gram-positive bacteria, including organisms from the genus *Bacillus* and *Clostridium*. For example, the organisms *Geobacillus (Bacillus) stearothermophilus* and *Bacillus pumilus* are routinely used as biological indicators in sterilization validation studies.

Many bacteria also form *capsules,* which are secreted extracellular polymers (primarily polysaccharides and some proteins) that are formed outside the bacterial cell walls. As fimbriae, capsules enable bacteria to attach to surfaces to form biofilms. In addition, because capsules are somewhat slimy structures, they help protect bacteria from phagocytosis and desiccation.

The Phyla Gram-Positive Bacteria and Proteobacteria

At least 40 distinct phyla from the Bacteria domain have been discovered [1], with various phenotypic variations among their members. Some of the key phyla are listed in Figure 1.1. In this chapter, we will focus on the Proteobacteria and Gram-positive bacteria because they contain most of the microorganisms of interest to the pharmaceutical microbiologist.

Gram-Positive Bacteria

The phylum Gram-Positive Bacteria contains several types of organisms with a common phylogeny and cell wall structure composed of several layers of peptidoglycan, in addition to small amounts of teichoic acid (see Figure 1.9). These organisms are divided into two major phylogenetic subdivisions: "low GC" and "high GC," where the GC ratio is used to determine the guanine (G) plus cytosine (C) content of an organism's genomic DNA. Included in this phylum are

- "Low-GC" endospore-forming bacteria *Bacillus* and *Clostridium*
- "High-GC" *Corynebacterium*, *Arthrobacter,* and *Propionibacterium*
- Nonsporulating "low-GC" genera *Staphylococcus*, *Streptococcus*, *Micrococcus*, and *Lactobacillus*
- "High-GC" filamentous *Streptomyces* and *Actinomyces*

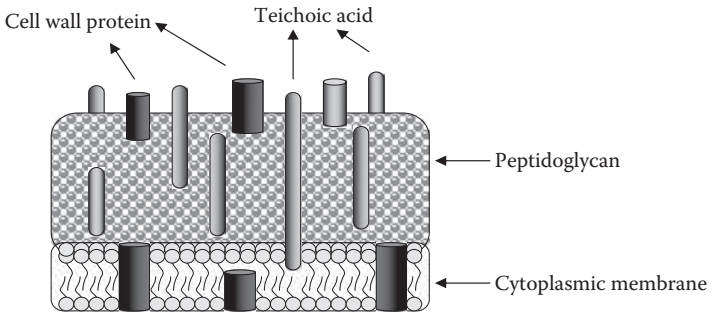


FIGURE 1.9 Cell wall composition of Gram-positive bacteria.

Mycoplasmas are phylogenetically related to "low-GC" Gram-positive bacteria despite the fact that they do not have a cell wall to enable them to retain the Gram stain.

Proteobacteria

The Proteobacteria is the largest phylum of Bacteria. All organisms are Gram-negative, and a large number of them are pathogens (Gram-negative rods and Gram-negative cocci). Proteobacteria have diverse metabolisms, including aerobes, facultative or obligate anaerobes, and heterotrophs—but there are also numerous exceptions. Some organisms are free-living and are responsible for nitrogen fixation in nature. Many proteobacteria are motile using flagella or gliding motion, whereas others are nonmotile. In Gram-negative bacteria, peptidoglycan is present in their cell wall in a much smaller amount as compared to Gram-positive organisms. However, besides peptidoglycan, Gram-negative bacteria contain an additional outer membrane layer composed of lipopolysaccharide (LPS; see Figure 1.10). *Endotoxin* is the term used to refer to this component of the LPS layer, in particular its Lipid A portion, which is toxic to humans and other animals, causing symptoms such as fever and diarrhea; in large doses, endotoxin can be fatal. Endotoxins are released only when cells are lysed or die, because LPS is part of the organism's cell structure. Therefore, a sample that may not be contaminated with viable Gram-negative organisms could in fact

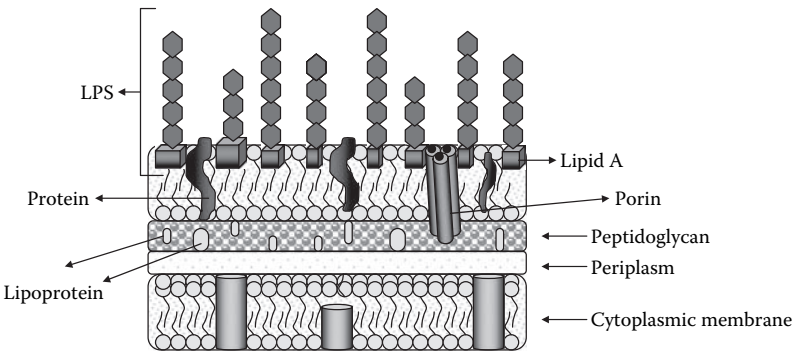


FIGURE 1.10 Cell wall composition of Gram-negative bacteria.

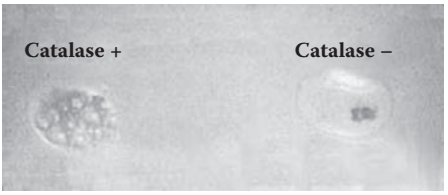


FIGURE 1.11 The catalase test reactions.

contain high concentrations of bacterial endotoxin; typical sterilization procedures that destroy viable bacteria do not remove endotoxins.

The Proteobacteria are divided into five subdivisions: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria. The two subdivisions of greatest significance to the pharmaceutical industry are the **Gammaproteobacteria**, which comprise organisms such as the Enterobacteriaceae, Pseudomonadaceae, and *Stenotrophomonas*, and the **Betaproteobacteria** , which comprise many environmental isolates found in waste water or soil, including species of the genera *Burkholderia, Comamonas,* and *Ralstonia*.

The Gram-Staining Method

The Gram stain, a technique that was perfected by the Danish bacteriologist Hans Christian Gram in 1884, is used to distinguish between Gram-positive and Gram-negative bacteria. In this procedure, bacterial cells are treated with a series of chemicals—crystal violet (stain), iodine (mordent), acetone-alcohol (decolorizer), and safranin (counter stain). The differences in the composition of the cell wall of bacteria, as described earlier, are responsible for the ability of some organisms to retain the crystal violet–iodine complex or not. The organisms able to retain the Gram stain appear purple in color and are classified as Gram positive. Gram-negative organisms are unable to retain the crystal violet–iodine complex during the decolorization procedure and appear red in color owing to the counter stain safranin. The Gram stain is a fast, simple, and very useful preliminary microbial identification tool to the pharmaceutical microbiologist. On the basis of the shape of the cell observed under the microscope and the Gram reaction, the microbiologist is able to narrow the identification of the unknown isolate and even determine the likely source of contamination. For example, many Gram-positive cocci, including *Staphylococcus epidermidis* and *Micrococcus* spp., are human borne whereas Gram-negative rods are typically associated with aqueous environments.

KOH Test

The potassium hydroxide (KOH) test was developed by Gregerson [5] to distinguish Gram-positive from Gram-negative rods. The protocol calls for aseptically removing a putative Gram-negative colony from a 24-h culture and stirring it up in a few drops of a 3% KOH solution on a clean glass slide and using a sterile loop. A few seconds later, the loop is slowly raised from the sample preparation to observe for viscous threads attached to the loop that are formed by cell wall lysis of Gram-negative rods and liberation of the viscid DNA. Gram-positive rods do not create viscous threads.

Catalase Test

Catalase is an enzyme that decomposes hydrogen peroxide into oxygen and water. Most aerobic and facultative anaerobic bacteria possess catalytic activity. The test is performed by transferring a portion of an isolated microbial colony to a clean glass slide. Then, a drop of dilute hydrogen peroxide solution (3%) is applied to the cells. Bacteria that produce catalase will convert the hydrogen peroxide, and oxygen will be evolved. Therefore, a positive reaction is indicated by the formation of gas bubbles. The reaction is negative if gas bubbles are not observed (see Figure 1.11). The catalase test is also used to differentiate streptococci (catalase negative) from staphylococci (catalase positive).

FUNGI

The phylum Fungi from the domain Eukarya comprises a large and diverse group of organisms that share some characteristics with both lower plants (algae) and lower animals, but are not closely related to either. Fungi are nonphototrophic and nonmotile microbes that are either unicellular or filamentous. There are three major groups of fungi: molds, yeasts, and mushrooms. In pharmaceutical microbiology, the focus is on yeasts (unicellular fungi) and molds (filamentous fungi).

The habitat of fungi is diverse, and most fungi prefer a dark and humid environment, i.e., relative humidity of 70% or higher [6]. Most fungi are found in the soil and on dead and decaying plant material. In fact, fungi have a significant role in biodegradation and recycling of organic matter. Some freshwater fungi are known, but only a few marine fungi have been reported. Yeasts are found in habitats with high sugar content and some are human borne.

Although there are about 250,000 fungal species, less than 150 have been reported to be human pathogens [7]. Most fungi are plant parasites. Yeasts are the most common human fungal pathogen, with the genus *Candida* having nearly 20 species described as human pathogens. The genus *Aspergillus* is the most common pathogen of the various filamentous fungi. However, other organisms from the genera *Fusarium*, *Penicillium*, *Alternaria*, and *Trichosporum* have also been recognized as opportunistic human pathogens. The dermatophytes *Trychophyton* and *Microspora* can digest keratin and thus have the potential to cause infections of keratinized tissues such as nails and hair.

In terms of environmental control, the presence of molds in manufacturing environments causes great concern because these types of organisms spread very rapidly since they produce spores that are easily transferred from one site to another. Many molds also produce mycotoxins and antibiotics, which can be a concern if these organisms are contaminants in products for human or animal consumption. Mycotoxins are products of secondary metabolism of molds whose primary function seems to be to give molds a competitive advantage over other mold species and bacteria. This is an important fact to know and understand when dealing with mold contamination issues: production of toxins and the types of toxins produced are very much dependent on the substrate and the competitive environment where the organisms are found. Therefore, the fact that a particular mold species was isolated from

a product sample does not in itself indicate that toxins were produced and product quality was impacted.

Cell Structures

Fungal cell walls are similar to plant cell walls in structure but not in chemical composition. The main constituent of a fungal cell wall is chitin and not cellulose. As eukaryotes, fungi contain true mitochondria and a nuclear membrane, but they lack chlorophyll and chloroplasts. Many fungi, except the yeast *Candida*, do not take up the Gram stain very well and may require special fungal stains to be observed under the microscope.

Fungal Growth and Reproduction

Fungi are chemoorganotrophs, and most have simple nutritional requirements. Fungi are mainly aerobic microorganisms, although there are species that are facultative anaerobes; no known true anaerobic fungi species have been identified to date. Fungi reproduce by both asexual and sexual means. Most fungi are mesophiles (optimum temperature range: 20–25°C), although some psychrophilic (e.g., *Cryptococcus albidus*) and even thermophilic fungi (e.g., *Hansenula polymorpha*) have been reported. The pH range for fungal growth varies from as low as 2.2 to as high as 9.6. However, most fungi prefer a pH environment around 5.0 or lower [8].

Molds

The filamentous fungi (molds) are so called because they form filamentous structures called *hyphae* (singular: hypha). When hyphae grow together, they form a structure called a mycelium (plural: mycelia), which is generally white and can be detected by the naked eye. As the mold matures, it develops spores (conidia) that are formed on the aerial branches of the hyphae (see Figure 1.12). Conidia are asexual spores that are somewhat resistant to drying; their main function is to spread the fungus to new habitats, a fact that makes these types of organisms common contaminants on most surfaces. Conidia are also highly pigmented and create the spectrum of colors so typical of molds: black, blue-green, red, yellow, and brown. Some molds also produce sexual spores that are more resistant to drying, heat, freezing, and even to some chemicals. Unlike bacterial spores, fungal spores are part of the normal life cycle of fungi, and, therefore, they are less resistant to chemicals and adverse environmental conditions.

Yeasts

Yeasts are unicellular fungi typically classified with the Ascomycetes. Yeast cells are generally round, oval, or cylindrical; under certain conditions, yeasts can form filaments. One example of this growth pattern is seen with *Candida albicans,* which expresses pathogenicity only in the filamentous form. Yeast colonies may appear similar to bacterial colonies on solid medium. However, under microscopic examination, yeast cells are much larger and can be easily distinguished from bacterial

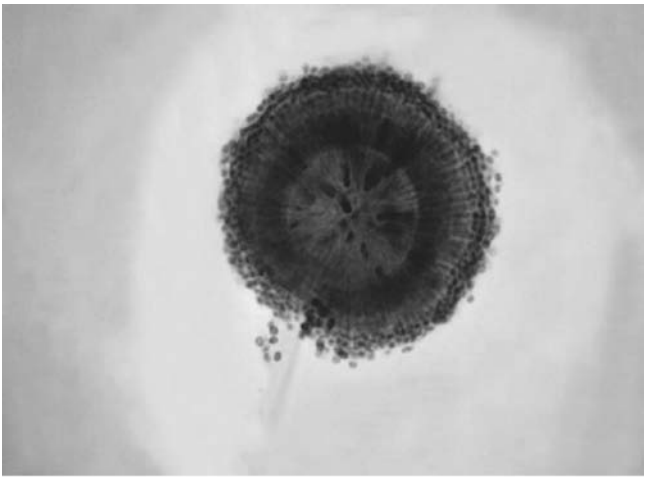


FIGURE 1.12 Conidial head of *Aspergillus niger.* (From Public Health Image Library, U.S. Department of Health and Human Services.)

cells. Yeast cells reproduce by budding or binary fission (see Figure 1.13). During budding, a small outgrowth (daughter cell) is formed, and it grows until it separates from the mother cell to become a separate cell. Some yeasts also undergo sexual reproduction by a process called *mating.*

MICROORGANISMS OF INTEREST

Included in the various pharmacopeias are requirements for absence of specified microorganisms considered objectionable because they represent a potential health hazard to the users. Some of these organisms are also referred to as "indicator" organisms because the ability to isolate and detect them using the given test methods provides good assurance that other similar potential pathogens, if present in the sample, would also be detected.

In addition to methods for total aerobic microbial count (TAMC) and total combined yeasts and molds count (TYMC), the harmonized methods in the compendia (USP, European Pharmacopoeia [EP], and Japanese Pharmacopeia [JP]) provide detailed tests for the detection of specified organisms, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp., Clostridia, bile-tolerant Gram-negative bacteria, and *Candida albicans.* These organisms are considered objectionable if present in certain types of products, depending on their known pathogenicity and/or contamination potential during pharmaceutical manufacturing. However, the presence of other organisms that might be considered objectionable to a product or manufacturing process must not be overlooked, a topic that will be further discussed in Chapter 2.

In this chapter, attention will be given to compendial organisms of concerns and other microorganisms that are typical contaminants in pharmaceutical/biopharmaceutical production facilities.

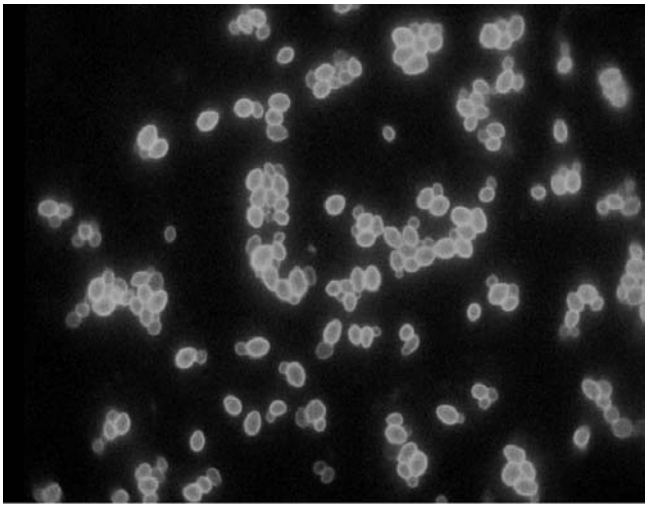


FIGURE 1.13 Oval budding yeast cells of *Candida albicans*. Fluorescent antibody stain. (From Public Health Image Library, U.S. Department of Health and Human Services.)

Genus *Staphylococcus*

Organisms from the genus *Staphylococcus* are Gram-positive bacteria from the family Staphylococcaceae. These organisms have spherical cells (cocci) about 0.5–1.5 μm in diameter. The cells occur singly, in pairs, or in tetrads. *Staphylococcus* organisms are nonmotile, facultative anaerobes, usually catalase positive, and most test negative for coagulase reaction. Columbia agar enhanced with colistin and nalidixic acid (CNA) is typically used as a selective medium for staphylococci as well as streptococci.

Staphylococci and other Gram-positive bacteria such as *Micrococcus* and *Streptococcus* are typical human-borne isolates and represent a large percentage of microorganisms isolated from environmental samples in pharmaceutical manufacturing facilities. The likely source of this type of contamination is inadequate aseptic technique and gowning practices, and poor clean room behavior.

Staphylococci cause infections of the skin and organs of the human body, including food poisoning. Toxins and enzymes produced by these organisms can also destroy red and white blood cells. Although coagulase-negative staphylococci are predominant members of the normal human skin flora and are generally considered nonvirulent, these organisms are also being increasingly recognized as opportunistic pathogens. Staphylococci are generally very resistant to current antibiotics, a fact that has become a challenge to treatment of nosocomial infections. An antibiotic-resistant form of *Staphylococcus aureus* caused a widespread infection in the Midwest of the United States in the late 1990s [9].

Some medically significant species of *Staphylococcus* organisms include *Staphylococcus aureus, Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, and *Staphylococcus haemolyticus*, the latter three species being mostly opportunistic pathogens and considered less virulent when compared to *Staphylococcus aureus.*

Staphylococcus aureus

The microorganism *Staphylococcus aureus* is listed in the compendia as an organism of concern for oral, nasal, inhalation, vaginal, and dermatological products. The major habitats of *Staphylococcus aureus* are the human skin, gastrointestinal tract, nasal membranes, and genital tract of warm-blooded animals. This known human pathogen causes a wide range of infections, including impetigo, toxic dermal necrolysis, pneumonia, meningitis, and toxic shock syndrome. Many strains of *Staphylococcus aureus* also produce enterotoxins that, if ingested, may produce symptoms of staphylococcal food poisoning.

Staphylococcus aureus can be isolated on a general microbiological medium such as TSA. On this medium, colonies are smooth, raised, glistening, circular, and translucent. Most strains have some type of pigment in their cell membranes, and colony coloration ranges from gray to grayish white, and from yellow-orange to orange. Colonies are gold or yellow on sheep blood agar, hence the given species name "aureus." *Staphylococcus aureus* are facultative anaerobes, but grow best under aerobic conditions. Most strains are catalase positive and produce coagulases, the latter being a key screening test in the routine differentiation of *Staphylococcus aureus* from other coagulase-negative species. The official method for detection of coagulases is the tube test method, which detects free coagulase. The slide test, which is a rapid test for the detection of clumping factor (coagulase bound to the cell wall), is recommended as a screening technique only, but it exhibits approximately 96% agreement with the tube method. A variety of plasmas may be used for either test, but rabbit plasma (dehydrated rabbit plasma containing citrate or ethylenediamine tetraacetic acid [EDTA]) is usually employed.

There are three types of selective media that can be used to screen for *Staphylococcus aureus:*

- Mannitol salt (MS) agar: This medium is used for the selective isolation of pathogenic staphylococci, because many other bacteria are inhibited by its high salt concentration (7.5%). Colonies of potentially pathogenic staphylococci are surrounded by a yellow halo that indicates mannitol fermentation. This medium is also listed in the official harmonized compendial method (USP Chapter <62>) for detection of *Staphylococcus aureus* from nonsterile products.
- Vogel–Johnson (VJ) agar: This medium is intended for the isolation of coagulase-positive, mannitol-fermenting *Staphylococcus aureus* organisms that form characteristic black colonies surrounded by a yellow zone owing to mannitol fermentation. The growth of other bacteria is almost completely inhibited by lithium chloride, high glycine concentration, and the presence of tellurite, a chemical that when reduced by pathogenic staphylococci yields a black precipitate in the colonies.
- Baird-Parker (BP) agar: This medium was originally formulated by Baird-Parker, and it has been recommended for use in the detection of *Staphylococcus aureus* by the EP and the FDA. It is intended for the isolation of coagulase-positive staphylococci after 24 h of incubation. The medium

contains lithium and potassium tellurite to suppress the growth of other types of organisms. Pyruvate and glycine are also added for the purpose of enhancing the growth of staphylococci. The tellurite and egg yolk components of the medium are responsible for the differentiation of coagulase-positive staphylococci by the formation of black, shiny, convex colonies surrounded by a clear zone from the coagulase-negative staphylococci.

Genus *Pseudomonas*

Organisms from the genus *Pseudomonas* are gammaproteobacteria from the family *Pseudomonadaceae,* which also includes the genus *Xanthomonas*. Both genera comprise the group of bacteria known as pseudomonads. The genus *Pseudomonas* contained over 200 species of organisms, but over the years the taxonomic heterogeneity of the genus was revealed, and during the last decade, certain members of this genus were reclassified into new genera. Many *Pseudomonas* organisms are plant pathogens, and several species are either known human pathogens or are considered opportunistic pathogens, especially for patients with compromised immune systems.

Pseudomonads are straight or slightly curved rods about 0.5–1.0 by 1.5–5.0 μm in size. They are Gram negative, motile by one or several polar flagella (rarely nonmotile), aerobic, oxidase positive or negative, and catalase positive.

These types of organisms are widely distributed in nature. Pseudomonads are common soil and water inhabitants and are often found in the intestinal tract of mammals and on plants. In pharmaceutical manufacturing environments, contamination with *Pseudomonas* organisms is usually associated with the presence of moisture: unattended stagnant water and wet floors, improperly drained drip pans, humidifiers, cooling towers, and sink traps.

Organisms from the genus *Pseudomonas* usually produce colonies on general microbiological media that have some color due to pigments. The fluorescent pigments pyoverdin and pyocyanin are their most notorious soluble pigments. Pyocyanin has been shown to induce cells to secrete interleukin-8 (IL-8), an important chemokine involved in cystic fibrosis (CF) inflammation. Therefore, pyocyanin has been targeted for new therapeutic approaches in treatment of CF patients. Most *Pseudomonas* species can grow in mineral media with a single organic compound as the sole carbon and energy source. These organisms prefer a medium with a pH close to neutrality, a good supply of oxygen, and an environment in the mesophilic range. However, *Pseudomonas* can survive and multiply in less than such an optimum condition; some pseudomonads are able to grow in distilled water if traces of organic matter are present.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a known human pathogen, and it is listed in the compendia as an organism of concern for oral, nasal, inhalation, vaginal, and dermatological products. These organisms are common contaminants of pharmaceutical liquid products because they are quite resistant to biocides. *Pseudomonas aeruginosa* is also the most frequently implicated member of the genus *Pseudomonas* in human

TABLE 1.1
Biochemical characteristics of *Pseudomonas aeruginosa*

Reaction	Result
Gelatin liquefaction	Positive
Oxidase reaction	Positive
Starch hydrolysis	Positive
Growth in nutrient medium at 4°C	Negative
Growth in nutrient medium at 42°C	Positive
Denitrification	Positive
Pyocyanin production	Positive
Pyoverdin production	Positive

infections of burn sites, wounds, urinary tract, and lower respiratory tract. Table 1.1 contains some of this organism's biochemical characteristics.

There are two main colony types of *Pseudomonas aeruginosa*. One is large and smooth with flat edges and an elevated center; the other is small, rough, and convex. The colonies also tend to spread and give off a characteristic grapelike odor caused by 2-aminoacetophenone. This organism has one flagellum and grows best at a temperature of 37°C. It utilizes glucose oxidatively in Oxidation-Fermentation medium, and it grows on medium containing cetyltrimethylamine bromide (cetrimide). Most other members of the genus *Pseudomonas*, except for *Pseudomonas fluorescens* and related nonfermentative organisms, are inhibited by this medium. Because *Pseudomonas aeruginosa* grows on eosin-methylene blue (EMB) and MacConkey agars as non-lactose-fermenting organisms, it is usually considered a suspicious enteric organism and thus transferred to Triple Sugar Iron (TSI) medium, which contains phenol red as pH indicator for further screening. Organisms that ferment lactose, such as *E. coli*, produce acid (yellow) slant and butt reactions with formation of gas. *Pseudomonas aeruginosa* produces alkaline (red) slant and butt reactions with no gas production.

The following are other typical media used for the isolation and detection of *Pseudomonas aeruginosa*:

- Cetrimide and Pseudosel agars: In these media, colonies of *Pseudomonas aeruginosa* are generally greenish. Fluorescence observed under ultraviolet light is greenish. Cetrimide is listed in the official harmonized compendial method (USP Chapter <62>), for detection of *Pseudomonas aeruginosa* from nonsterile products.
- *Pseudomonas* agars: These media are designed for the detection of fluorescein and pyocyanin. Observed under ultraviolet light, *Pseudomonas aeruginosa* yields the following:
  - *Pseudomonas* fluorescein agar: Colonies are generally colorless to yellowish; fluorescence observed under ultraviolet light is yellowish.
  - *Pseudomonas* pyocyanin agar: Colonies are generally greenish; fluorescence observed under ultraviolet light is blue.

Another typical test performed to confirm the presence of *Pseudomonas aeruginosa* is the oxidase test. This procedure involves the transfer of suspect colonies to oxidase strips, disks, paper/reagent, or swabs. The development of a pink color changing to purple indicates a positive reaction, which is the expected result for confirmation of putative *Pseudomonas aeruginosa* organisms.

Genus *Burkholderia*

Organisms from the genus *Burkholderia* are betaproteobacteria from the family Burkholderiaceae. This genus comprises several well-known human pathogens, including *Burkholderia cepacia*, *Burkholderia maltei*, *Burkholderia pseudomalei*, and *Burkholderia gladioli*. *Burkholderia* organisms are Gram-negative straight or slightly curved rods, motile, aerobic, oxidase positive or negative, and catalase positive. Given the numbers of incidents of contamination of pharmaceutical products with *Burkholderia cepacia*, this organism has been given further attention by the regulatory agencies and pharmaceutical companies and therefore will be discussed in further detail in this chapter.

Burkholderia cepacia

Burkholderia cepacia and *Pseudomonas aeruginosa* have very similar metabolisms. However, at the genetic level these organisms are very different; thus, the removal of the species *cepacia* from the genus *Pseudomonas*. *Burkholderia cepacia* is a versatile microbe found in a variety of aqueous environments and recognized as a useful organism for plant protection from other pathogens. However, *Burkholderia cepacia* is also a life-threatening human pathogen usually associated with respiratory infections of CF patients. In recent years, even non-CF patients have succumbed to this organism in intensive care units. Unlike *Pseudomonas* organisms, *Burkholderia cepacia* can be easily spread from patient to patient, and it is resistant to all aminoglycoside drugs, which are typically used to treat *Pseudomonas aeruginosa* infections. Monitoring and controlling contamination of facilities, clean utilities, and products for the presence of *Burkholderia cepacia* have become priorities for certain manufacturers of drugs that are at risk of being contaminated with this known pathogen.

The following are some of the selective media that can be used to screen for *Burkholderia cepacia*:

- *Burkholderia cepacia*–selective agar (BCSA; Dalynn Biologicals, www.dalynn.com): Colonies on this medium are greenish brown with yellow halos. The yellowing of the medium is due to carbohydrate fermentation, but it may not occur with every isolate of this species. Other organisms such as *Enterococcus faecalis* and *Ralstonia pickettii* may also grow on BCSA, but growth of *Pseudomonas aeruginosa* is inhibited.
- *Burkholderia cepacia* agar (PML Microbiologicals, www.pmlmicro.com): Microbial colonies on this medium develop a pink to hot pink color; isolated colonies of *Burkholderia cepacia* appear gray to white in color; size varies from pinpoint to 2 mm and a purplish hue may develop.

- MacConkey agar: Putative colonies that are smooth, yellow, or white and Gram stain negative should be transferred to TSI slants for further characterization. *Burkholderia cepacia* gives an alkaline reaction in the slant and the butt.

Genus *Ralstonia*

Organisms from the genus *Ralstonia* are betaproteobacteria from the family Ralstoniaceae. These are Gram-negative aerobic motile rods isolated from plants, soil, and water. The genus *Ralstonia* was proposed in 1995 on the basis of phenotypic characterization, phylogenic analysis of 16S rRNA nucleotide sequences, and rRNA DNA hybridization [10]. Since its creation, the taxonomy of the genus has expanded to include 11 species, including *Ralstonia pickettii*, a typical water system contaminant and organism of concern to the pharmaceutical industry owing to its potential for contaminating liquid products. *Ralstonia* share many similarities with *Burkholderia,* but the organisms from these two genera can be distinguished on the basis of assimilation of galactose, mannitol, mannose, and sorbitol [10].

Ralstonia pickettii

Since the early 1970s, *Ralstonia pickettii* (formerly *Pseudomonas pickettii/Burkholderia pickettii*) has been isolated from contaminated pharmaceutical solutions such as sterile Modudose® 0.9% saline solution and intravenous Ranitidine, which were associated with respiratory and blood infections [11]. In fact, *Ralstonia pickettii* has become a microorganism of concern to sterile drug manufacturers that rely on traditional 0.2-µm sterilizing-grade filters to render their solutions sterile because this organism is capable of passing through these types of membrane filters [12].

Ralstonia pickettii can be isolated using general media for heterotrophic organisms. Microbiologists often fail to differentiate and isolate it from other closely related species, such as *Burkholderia cepacia,* on the basis of colonial morphology.

Ralstonia pickettii is currently not on the list of compendial organisms of interest. However, companies that produce pharmaceutical solutions and other liquid products should consider controlling *Ralstonia pickettii* and preventing it from contaminating their water systems. Designing methods for water testing and products that are capable of screening for and detection of *Ralstonia pickettii* may be needed and should be considered based on a risk management approach.

Genus *Comamonas* and Genus *Stenotrophomonas*

Comamonas organisms are betaproteobacteria from the family Comamonadacea, whereas *Stenotrophomonas* organisms are gammaproteobacteria from the family Xanthomonadaceae. These Gram-negative organisms are isolated from soil, animals, and plants, and can be opportunistic human pathogens. *Stenotrophomonas maltophilia*, formerly known as *Pseudomonas maltophilia* and *Xanthomonas maltophilia*, has recently emerged as an organism of concern in the hospital environment, primarily owing to the increase in the number of immunocompromised patients. However, according to the most recent reports, this organism has not been

associated with infections of healthy individuals. *Comamonas avenue*, *Comamonas tarragona*, and *Comamonas testosterone* have all been associated with infections such as bacteremia and conjunctivitis. Organisms from the genera *Comamonas* and *Stenotrophomonas* are currently not considered compendial organisms of interest. However, because both of these organisms have been associated with biofilm formation in pharmaceutical-grade water systems and bioprocessing equipment and, as Gram negatives, they are endotoxin producers, companies should evaluate whether or not such organisms should be considered objectionable to their manufacturing processes.

Family Enterobacteriaceae

Members of the Enterobacteriaceae family are gammaproteobacteria Gram-negative straight rods, motile or nonmotile; they do not form endospores and are not acid fast. During fermentation of D-glucose, other carbohydrates, and polyhydroxyl alcohols, acid and gas are produced. Enterobacteria are catalase positive and oxidase negative (except for *Shigella dysenteriae* 0 group 1 and *Xenorhabdus nematophilus*). These organisms can be found in soil, water, plants, and animals. *Escherichia coli* and *Salmonella* spp. are organisms from the Enterobacteriaceae family listed in the compendia as organisms of concern, especially for oral products. These organisms are not typical environmental contaminants in pharmaceutical manufacturing facilities unless there are inadequate waste/sewage systems in place.

Enterobacteria are well known for their rapid generation time, and the following are enrichment media listed in the pharmacopeias for their isolation:

- Mossel enterobacteriacea enrichment broth (EE broth): Used as an enrichment medium for the isolation of bile-tolerant Gram-negative bacteria.
- Violet red bile (VRB) agar with glucose and lactose: Used for the detection and enumeration of enterobacteria. VRB agar contains lactose. By adding glucose, the recovery of glucose-fermenting enterobacteria is improved, which is evidenced by formation of red colonies with red-purple halos.

Genus *Escherichia*

For many years, the genus *Escherichia* had been represented by the single species *Escherichia coli*. However, with the taxonomic reorganizations of the last two decades, four new species were added to the genus: *Escherichia hermannii, Escherichia vulneris, Escherichia fergusonii,* and *Escherichia blattae,* the latter having been isolated from nonhuman sources only. *Escherichia coli* remains the most studied and medically significant species of the genus. It is listed in the compendia as a representative Gram-negative fermenting bacterium to be used in method validations, antimicrobial challenges, and quality control of media.

Escherichia coli

Escherichia coli organisms are facultative anaerobes, occurring singly or in pairs of straight rods of about 1.1–1.5 by 2.0–6.0 μm in size. Both motile and nonmo-

TABLE 1.2
Biochemical characteristics of *Escherichia coli*

Reaction	Result
Gelatin liquefaction at 22°C	Negative
H_2S on TSI	Negative
D-Glucose gas production	Positive
Lactose fermentation	Positive
Oxidase reaction	Negative

tile forms occur in nature. Its habitat is the lower part of the intestine of warm-blooded animals. Its presence in water, raw materials, and products is indicative of fecal contamination and a lack of hygiene in production areas. When found as facility isolates, it can be an indication of inadequate/uncontrolled personnel traffic (feet contamination originating from restrooms), or inadequate sanitization procedures, especially in biotechnology facilities that use this organism in fermentation processes.

Escherichia coli is typically differentiated from other members of the Enterobacteriaceae family by its rapid fermentation of lactose with acid and gas production. Its optimum growth temperature is 37°C. Colonies on nutrient agar are smooth, low convex, moist, and gray, or they may be rough and dry.

Escherichia coli is an opportunistic pathogen, and some strains produce enterotoxins that can cause fever reactions. *Escherichia coli* has been associated with a number of diseases, including cystitis, appendicitis, gallbladder infections, septicemia, meningitis, endocarditis, and epidemic diarrhea.

Selective agars used for detection of *Escherichia coli* usually contain chemicals such as tetrathionate, desoxycholate, and bile salts to inhibit the growth of nonenteric organisms. However, an all-purpose medium, such as TSA or blood agar, can also be used for the isolation of these organisms; most strains are nonhemolytic. Table 1.2 contains typical biochemical reactions produced by *Escherichia coli*. The following are the selective media listed in the pharmacopeias for detection of *Escherichia coli*:

- MacConkey (MAC) agar: This medium is inhibitory for Gram-positive bacteria. Coliforms that are lactose fermenting produce red colonies, and non–lactose-fermenting organisms produce colorless colonies. The agar concentration can be increased to 5% to inhibit the spread of organisms from the genus *Proteus*.
- Eosin–methylene blue (EMB) agar: On this medium *Escherichia coli* forms colonies with a characteristic metallic sheen under reflected light and blue appearance under transmitted light. Levine EMB does not contain sucrose. If a sucrose-containing medium is used instead, *Proteus* spp. also produce a metallic sheen. In such cases, the agar concentration in the medium must be increased to 5% in order to inhibit growth of *Proteus* organisms. EMB agar

also allows for the distinction between lactose-fermenting and non–lactose-fermenting bacteria.

Genus *Salmonella*

Organisms from the genus *Salmonella* are mostly aerogenic (gas producing) motile Gram-negative straight rods about 0.7–1.5 by 2.0–5.0 µm in size. They conform to the general definition of the family Enterobacteriaceae, and they are closely related to *Escherichia coli*. As facultative anaerobes, they reduce nitrate to nitrites and usually produce gas from glucose and hydrogen sulfide (H_2S) in TSI. (A few species, such as *Salmonella choleraesuis* and *Salmonella paratyphi* A, do not produce H_2S.) Citrate is often used as the sole carbon source, and they do not ferment lactose. These organisms yield a positive reaction with methyl red, a negative reaction for gelatin liquefaction at 22°C, and a negative oxidase test.

Most *Salmonella* spp. are pathogenic to humans, causing enteric fevers, gastroenteritis, and septicemia. *Salmonella typhimurium* and *Salmonella enteriditis* are both known to cause food poisoning. *Salmonella* organisms may also infect other animals besides humans. If present in oral pharmaceutical formulations, these organisms cause nausea, vomiting, and diarrhea. Most *Salmonella* species are host adapted and isolated from intestinal tracts of animals. In a pharmaceutical environment, these organisms represent a contamination from sources such as raw materials of natural source, animal products, and soil. Given the fact that most *Salmonella* species are human pathogens, any organism from the genus *Salmonella* is considered objectionable.

The following are the selective media listed in the pharmacopeias for the detection of organisms from the genus *Salmonella*:

- Deoxycholate citrate agar (DC): This medium contains lactose, neutral red, and the selective chemical sodium deoxycholate. *Salmonella* spp. appear as well-developed colorless colonies.
- *Salmonella-Shigella* agar (SS): This medium contains lactose, neutral red, and the selective chemicals brilliant green and bile salts. Ferric citrate is also present as an indicator of H_2S production. This medium inhibits the growth of coliform bacilli.
- Brilliant green agar (BG): This medium contains lactose, phenol red, and brilliant green. In this medium, lactose-fermenting organisms form green colonies. *Salmonella* spp. form small, transparent, colorless or pink to white opaque colonies, frequently surrounded by a pink to red zone.
- Bismuth sulfite agar (BS): This medium is highly recommended for the isolation of *Salmonella typhi* and other *Salmonella* species. *Salmonella* spp. form black or green colonies.
- Xylose-lysine-desoxycholate agar (XLD): This medium is used for the screening of enteric pathogens, especially *Shigella* species. Colonies appear red with or without black centers.
- TSI slants: In this medium, pH changes occur after 18–24 h of incubation. S*almonella* organisms will turn the TSI slant red (alkaline) and the butt

yellow (acid), with or without H_2S production. The following are chemical reactions observed on TSI slants:

- Acid butt (yellow) and alkaline slant (red) indicate glucose fermentation.
- Acid throughout medium (yellow) indicates lactose and glucose fermentation.
- Gas bubbles in butt or medium sometimes split indicates aerogenic culture.
- Blackening in butt indicates H_2S production.
- Alkaline throughout medium (red) indicates none of the three sugars are fermented.

- Rappaport Vassiliades *Salmonella* enrichment broth: This is an alternative medium to fluid selenite cystine and tetrathionate broths for the isolation and detection of *Salmonella* spp.
- Hektoen enteric agar: this medium is used for isolation and detection of *Salmonella* spp., which appear as blue-green colonies with or without black centers.

Genus *Shigella*

Organisms from the genus *Shigella* are Gram-negative motile rods that are pathogens of humans and other primates, causing bacillary dysentery. They ferment sugars without gas production, and they do not use citrate or malonate as the sole carbon source. These organisms do not grow in potassium cyanide (KCN) or produce H_2S. On the basis of DNA relatedness analysis, members of the genus *Shigella* should be included in the genus *Escherichia*. However, they remain apart only because of historical reasons. As a matter of fact, *Escherichia coli* and *Shigella* strains (except *Shigella boydii* serovar 13) are indistinguishable on the basis of DNA hybridization studies.

For isolation and detection of *Shigella* spp., either MacConkey, XLD, or EMB agar should be used in combination with DC agar or *Salmonella-Shigella* agar.

Genus *Serratia*

As members of the Enterobacteriaceae family, *Serratia* organisms are short, motile, Gram-negative rods. They are isolated from soil and water, and although preferring a temperature range of 30–37°C, some strains can grow under refrigerated conditions. *Serratia* organisms are responsible for localized infections of wounds and of the urinary and respiratory tracts in humans.

Genus *Klebsiella*

Organisms from the genus *Klebsiella* are members of the Enterobacteriaceae family. They are short, aerobic, nonmotile Gram-negative organisms. They often form encapsulations made of carbohydrates. They ferment carbohydrates with acid and gas production. They can be found in soil, water, and the intestinal and respiratory tracts of animals, including humans. *Klebsiella pneumoniae* is one of most notorious pathogens from this genus, causing severe human infections.

Currently, organisms from the genera *Shigella*, *Serratia*, and *Klebsiella* are not specifically listed in the compendia as being organisms of concern. However, given their pathogenicity, it is prudent to treat the isolation of enterobacters and other nonenteric Gram-negative organisms as objectionable organisms depending on where these organisms are isolated from.

Genus *Bacillus*

The genus *Bacillus* belongs to the family Bacillaceae in the class Bacilli. These are Gram-positive organisms with rod-shaped and straight cells that may occur singly or in chains. Cell size varies from 0.5 by 1.2 μm to 2.5 by 10 μm. *Bacillus* organisms are able to form endospores that are resistant to most adverse conditions, including desiccation. However, they are not able to produce spores under anaerobic conditions. Although *Bacillus* spp. are Gram-positive organisms, older cultures can lose their Gram-staining positivity and may give a Gram-negative reaction. The KOH test developed by Gregersen [5] and described earlier in this chapter can be a useful tool to confirm whether the organism is indeed Gram-negative or Gram-positive.

Several species of *Bacillus* produce carbohydrate capsules. Some species tend to form spreaders on moist solid media. Most species are unpigmented and are beta-hemolytic. *Bacillus* organisms are motile by means of flagella, and are aerobic or facultative anaerobic. These organisms exhibit a wide variety of cell morphology, growth conditions, and nutritive requirements. As far as temperature and pH are concerned, requirements range from psychrophilic to thermophilic, and from acidophilic to alkaliphilic, respectively. *Bacillus* organisms are catalase positive (most species), but can be either oxidase positive or negative.

Organisms from the genus *Bacillus* are widely distributed in nature. However, their primary habitats are the soil, runoffs, dust, and infected plant materials. Spore-forming organisms from the genera *Bacillus* and *Paenibacillus* are common isolates from pharmaceutical environments, including air and surfaces, often as a result of feet/wheel contamination. Some species are human pathogens, such as *Bacillus anthracis* and *Bacillus cereus*. Other species may be opportunistic pathogens: *Bacillus cereus, Bacillus subtilis, Bacillus sphaericus, Bacillus circulans*, and *Bacillus pumilus* have all been associated with cases of meningitis, pneumonia, septicemia, and endocarditis.

Most methods for the detection and isolation of members of the genus *Bacillus* involve evaluation of the resistance of their spores to elevated temperatures. These organisms are very resistant to most disinfectants and to heat treatments at 70°C or above for 10 min or longer. Species of extremely thermophilic non–spore-forming *Bacillus* also survive this type of heat treatment.

Currently, there are no species of *Bacillus* organisms listed in the pharmacopeias as organisms of concern. However, given the fact that many *Bacillus* organisms can be opportunistic human pathogens, such as *Bacillus cereus*, in some cases, the microbiologist may have to decide whether an isolate should be treated as an objectionable organism or not, depending on product type, bioburden level, route of administration, manufacturing process, and target patient population.

Bacillus subtilis

Bacillus subtilis is listed in the compendia as an indicator organism, representative aerobic spore-forming Gram-positive rod, to be used in method validations, antimicrobial challenge tests, and quality control testing of media. This organism is found seldom in chains and with widespread endospores. Colonies are round or irregular, dull, and cream to brown colored and most are wrinkled. It forms spreaders on moist solid media. A reddish pigment may be formed under the colonies. It grows actively at a pH range of 5.5–8.5.

Bacillus cereus

This organism is a facultative anaerobe with widespread spores and often found in chains. Colonies are dull or with the appearance of frosted glass. On nutrient agar, some strains darken the medium, whereas others produce a pinkish brown pigment. When present in a pharmaceutical environment, it indicates dust contamination. This organism may cause food poisoning. *Bacillus thuringiensis* can be distinguished from *Bacillus cereus* by the production of a protein parasporal crystal in the cell.

Genus *Clostridium*

The genus *Clostridium* belongs to the family Clostridiaceae in the class Clostridia. Organisms from this genus are spore-forming, catalase-negative, Gram-positive rods that come singly or in pairs, in short or long chains, some even in tight coils. They can be motile or nonmotile, and may have oval or spherical endospores. *Clostridium* spp. have a wide variety of nutritional and growth requirements. Organisms can be saccharolytic, proteolytic, neither, or both. Most species are obligate anaerobes. They grow at a wide temperature range of 15–69°C. However, these organisms obtain the most optimum growth at a pH of 6.5–7.0 and at a temperature range of 30–37°C. In mixed cultures, they can be isolated using a 1-h exposure to a 50% ethanol solution or by heat shock at 80°C for 10 min to isolate the spore-forming cells. Sample preparations are then struck onto blood agar or egg yolk agar and incubated anaerobically. Alternatively, the heated sample preparations can be cooled rapidly, subcultured with reinforced medium for Clostridia and further incubated at 35–37°C for a minimum of 48 h under anaerobic conditions. After incubation, the sample preparation is subcultured onto Columbia agar and further incubated 35–37°C for another 48 h under anaerobic conditions. The presence of *Clostridium* species is confirmed by microbial growth of catalase-negative Gram-positive rods.

Some species of the genus *Clostridium* are pathogenic to humans and animals. Their toxins can cause tetanus and botulism. *Clostridium* spp. are commonly found in soil, dust, sewage, marine sediments, and decaying vegetation or animal products. They can also be found in the intestinal tract of animals and in wounds. Because of their widespread presence in nature, they are potential contaminants of pharmaceutical products.

Because of the known pathogenicity of many *Clostridium* spp., these organisms are considered objectionable. A test for detection of Clostridia is included in the

harmonized compendial tests for specified organisms (USP Chapter <62>). The following are typical media used for the detection of *Clostridium* species:

- Reinforced medium for Clostridia: This broth is supplemented with the enzyme oxyrase to create a suitable anaerobic environment for isolation and detection of Clostridia.
- Columbia agar: This is a general nutrient agar used for the cultivation of fastidious and nonfastidious organisms. Sheep or horse blood is added to detect hemolytic reactions of fastidious microorganisms. Recovery of Clostridia is accomplished under anaerobic conditions. The catalase test is used to confirm isolation of *Clostridium* organisms, which are catalase negative.

The EP also provides a test for the determination of *Clostridium perfringens* count. In addition, *Clostridium sporogenes* and *Clostridium perfringens* are two compendial indicator organisms typically used in method validations and quality control testing as representative anaerobic spore-forming Gram-positive rods.

Clostridium perfringens

This organism is a nonmotile Gram-positive rod that is able to produce acid from lactose. Most strains have a capsule composed of polysaccharides. Colonies on blood agar are 2–5 mm in diameter, circular, gray to grayish yellow, and with a glossy surface; it usually forms a characteristic double zone of hemolysis, with the inner zone showing complete hemolysis and the outer zone showing incomplete hemolysis. Presence of *Clostridium perfringens* in lactose sulfite medium is indicated by blackening of the medium and gas production.

Clostridium perfringens produces several soluble substances that cause toxic effects and possible virulence, such as gas gangrene, necrotic enteritis, and food poisoning. It is the species from the genus *Clostridium* that is most commonly isolated from infections in humans.

Clostridium sporogenes

This organism is a motile Gram-positive rod that sporulates readily on most media. Its spores are oval and subterminal. Surface colonies on blood agar are 2–6 mm in diameter, irregular, coarse with rhizoid edges, and with a yellowish to gray center usually forming visible filaments. This organism is able to yield good growth in 100% carbon dioxide (CO_2) atmosphere, and it grows best at a temperature range of 30–40°C. *Clostridium sporogenes* is able to produce ammonia and H_2S, and reduce neutral red. It is found throughout the world, from marine and freshwater sediments to feces of animals, including humans. *Clostridium sporogenes* is also found in human polymicrobial infections and is thought to promote invasiveness of other bacteria. It causes infective endocarditis and abscesses in wounds. This organism can be differentiated from closely related species by gas chromatographic analysis of cell wall fatty acids.

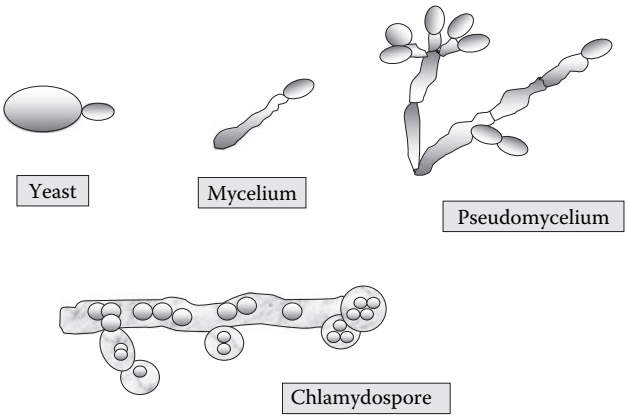


FIGURE 1.14 Morphologies of *Candida albicans*.

Candida albicans

This yeast organism belongs to the order Cryptococcales, a heterogeneous group of organisms thought to be the imperfect stages of and representing the asexual phase of ascomycetous and basidiomycetous yeasts. It is a dimorphic organism usually found in the yeast phase. With age, it forms chlamydospores that are more resistant to adverse environmental conditions. This organism can also be induced to form pseudo or true mycelium if the right conditions are present in the enrichment medium. For example, on sabouraud dextrose agar or potato dextrose agar (PDA), the yeast form (Form One) is detected. However, if the organism is cultured on cornmeal agar, rice cream agar, or chlamydospore agar, pseudomycelium (Form Two) and mycelium (Form Three) are produced. On these special media, chlamydospores (Form Four) also appear. This form of the organism is produced from pseudomycelium after a delay of 24 h. See Figure 1.14 for a depiction of the four forms of *Candida albicans*.

In the Compendia, *Candida albicans* is a pathogen often isolated from warm-blooded animals, including humans, where it exists as part of the normal flora of mucous membranes. It can also be isolated from many different sources, such as water, soil, plant and animal materials, and animal feces. *Candida albicans* causes many forms of candidiasis in humans, such as oral and vaginal thrush.

Candida albicans is the only pathogenic yeast species screened for and also used as an indicator organism in method validations and quality control testing.

As mentioned earlier, *Candida albicans* can be isolated and detected using all-purpose fungal media. It grows best in a moist environment at a temperature range of 20–25°C. Colonies are large, whitish, round, and moist (Figure 1.15). Production and detection of chlamydospores is used as a test to distinguish *Candida albicans* from other related species.

The following are media that can be used for the detection of *Candida albicans*:

- Sabouraud dextrose agar: This medium is recommended in the USP for isolation of *Candida albicans*. Growth of white colonies indicates possible

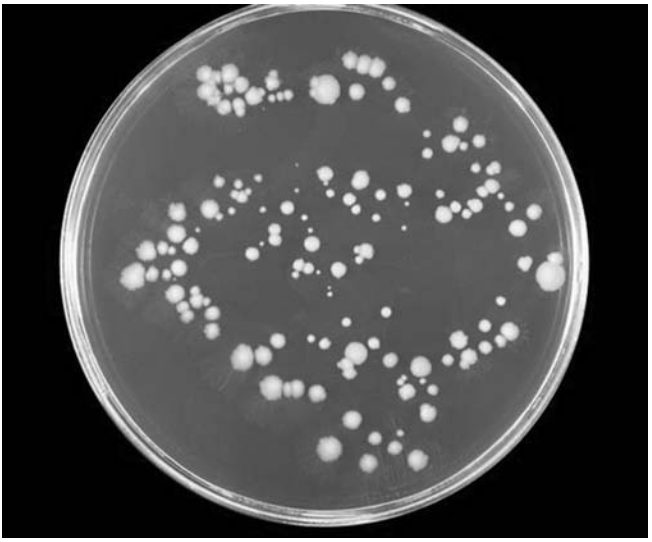


FIGURE 1.15 Plate culture of *Candida albicans*, strain 7H10, grown at 37°C. (From Public Health Image Library, U.S. Department of Health and Human Services.)

presence of *Candida albicans* organisms that must be confirmed by suitable identification tests.

- Cornmeal agar: This medium is used for the isolation and detection of general fungi. On this medium, *Candida albicans* forms filamentous colonies (pseudomycelia and mycelia) with formation of chlamydospores that can be detected by microscopic examination.
- Chlamydospore agar: This is a selective medium for *Candida albicans*; on this medium, this organism forms filamentous colonies with accumulation of tryptan blue in chlamydospores.

Zygosaccharomyces rouxii

Zygosaccharomyces rouxii, originally called *Saccharomyces rouxii*, is a yeast organism in the family Saccharomycetaceae. The *Saccharomyces* genus was reclassified to Zygosaccharomyces in 1983 [13]. Organisms from this genus are very resistant to many common preservation methods used in the food industry, making these yeasts common contaminants of food products. For example, *Zygosaccharomyces rouxii* is a nonpathogenic yeast that can cause product spoilage in pharmaceutical and food products having low-water activity and high sugar content. In the EP, screening for this organism is recommended for oral products containing a high sugar concentration.

Genus *Aspergillus*

Organisms from the genus *Aspergillus* belong to the subdivision Deuteromycotina. Aspergilli are filamentous fungi and among the most common fungal contaminants in pharmaceutical manufacturing environments. Because these organisms produce a wide variety of enzymes, they can grow practically everywhere and utilize many

different substrates. Some species also produce mycotoxins, aflatoxin being the most significant of them. *Aspergillus flavus* is the species responsible for the production of aflatoxins that causes fatal diseases in many farm animals. A related aflatoxin called carcinogenic aflatoxin B is thought to cause liver cancer in humans and other animals.

The color of the microbial growth is predominantly due to the color of the conidiophores and conidia that are produced in abundance. Pigment production in *Aspergillus* is influenced by the presence of trace elements. Aspergilli grow best at a temperature range of 20–25°C in a low-pH medium containing sugars such as dextrose.

Aspergillus niger

The black aspergilli, or *Aspergillus niger*, is widely distributed from the arctic regions to the tropics. Conidia from this organism can be found in the air and soil everywhere. Microbial growth starts with a white mycelium that turns yellowish and then quickly turns black owing to the spores on the conidia. *Aspergillus niger* grows best at 25°C; however, it is also able to grow in a wide temperature range up to 50°C. This organism is known to cause aspergillosis of the lungs (black lung disease). Because it can grow almost anywhere, it causes discoloration and spoilage of products and packaging materials.

Aspergillus niger is a compendial mold indicator organism also used in method validations, antimicrobial challenge tests, and media quality control testing.

Genus *Penicillium*

Penicillium organisms are as common as aspergilli, but not as pathogenic. They are the so-called blue and green molds. Their conidia can be found everywhere. Their significance in a pharmaceutical environment is due to their capability and potential to spoil pharmaceutical products and packaging by discoloration, and by the production of penicillins that can cause adverse reactions in sensitized patients. Some species of *Penicillium* have been associated with animal and human diseases.

In a pharmaceutical environment, both *Aspergillus* and *Penicillum* organisms are common air and surface contaminants in areas with inadequate humidity control, lack of high-efficiency particulate air (HEPA) filtration, inappropriate sanitization practices of materials and equipment transferred to manufacturing suites, and in areas where there is presence of water damage or leaks.

For more detailed information on the ecology, physiology, and detection of the compendial indicator and specified organisms as well as other organisms of concern, the author recommends the following reference books: Bergey's *Manual of Systematic Bacteriology* (2001), *Pharmaceutical Microbiology* (Hugo and Russell, 1992), and *Brock Biology of Microorganisms* (10th Edition, 2003).

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2 Microbial Contamination and Control

MICROBIOLOGICAL CONTAMINATION

Pharmaceutical products are subject to microbiological contamination that can represent a health hazard to the consumer and cause product spoilage, aesthetic changes, and possible loss of drug efficacy. Microbial contamination may originate from the raw materials and excipients or may be introduced during manufacture (operators and contaminated equipment, environment, and packaging materials), storage, and use. Most raw materials used in pharmaceutical manufacturing, including water, may contain several types of microorganisms. Depending on the type of the manufacturing process, these contaminants may be reduced or eliminated. However, care must be taken not to further increase the potential for introducing microorganisms during an uncontrolled manufacturing process.

The microbial contamination of pharmaceuticals has been studied extensively during the past 20 years. The products most prone to microbial contamination are those with a moderate to high water activity and availability, products containing sweeteners and sugars, and products in multidose containers. This is one of the main reasons that preservative systems are added to these types of drug formulations.

Product quality is evaluated through examination and monitoring of the conditions and practices in the facilities where they are manufactured, packaged, stored, and tested as well as through sampling and analysis of the finish dosage forms. Products that are found to be contaminated with microorganisms are recalled from the market. A product can also be recalled if there is evidence that a deviation occurred during its manufacture or distribution, resulting in a possible risk to public health. Such incidences typically occur in small numbers of batches. However, if a product is found to be unsafe for continued marketing, it must be withdrawn completely.

Product Recalls

According to the Center for Drug Evaluation and Research (CDER) reports to the nation on drug safety and quality, there were 401 prescription and 101 over-the-counter (OTC) drug recalls in the fiscal year of 2005. One firm alone had more than 100 products recalled, which resulted in the spike for the recall figures (refer to Figure 2.1). Out of the top 10 reasons for the recalls in 2005 by the FDA microbial contamination of nonsterile products was listed as number three. Other reasons given were as follows:

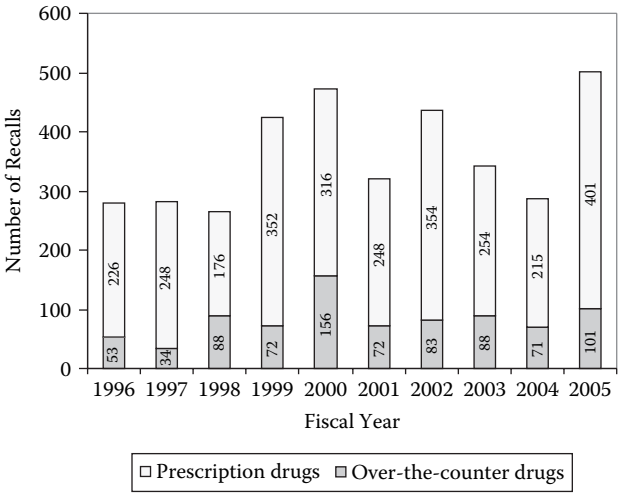

FIGURE 2.1 Prescription and OTC drug recalls. (From www.fda.gov/cder/reports/rtn/2005/rtn2005-4.htm.)

- Miscellaneous current good manufacturing practices (cGMP) deviations (other than the ones listed in the following text)
- Failed USP dissolution test requirements
- Lack of efficacy
- Impurities/degradation products
- Lack of assurance of sterility
- Lack of product stability
- Labeling: Label error on declared strength
- Misbranded: Promotional literature with unapproved therapeutic claims
- Labeling: Correctly labeled product in incorrect carton or package

In fact, microbial contamination has been on the list for top 10 reasons for FDA product recalls for the past 5 years with the most commonly detected organisms found in aqueous formulations being pseudomonads and other Gram-negative organisms. The following are some examples of recent safety advisories and product recalls issued by the FDA Safety Information and Adverse Event Reporting System (AERS):

- January 2008: Eye Drops and Eye/Ear Wash (NuCel Labs of Idaho Falls, Idaho). Voluntary nationwide recall due to product contamination with bacteria and particulate matter. Eye drops found to be contaminated with microorganisms can have serious effects, including blindness.
- January 2008: Sierra Pre-Filled Inc. and B. Braun Heparin and Saline prefilled syringes (AM2 PAT Inc., Angier, North Carolina). Heparin IV flush syringes were found to be contaminated with *Serratia marcescens*, which resulted in patient infections. The Centers for Disease Control and Prevention (CDC) confirmed growth of *Serratia marcescens* from several unopened syringes of this product.

- December 2007: PedvaxHIB® [Haemophilus b Conjugate Vaccine (Meningococcal Protein Conjugate)] and certain lots of COMVAX® [Haemophilus b Conjugate (Meningococcal Protein Conjugate) and Hepatitis B (Recombinant) Vaccine (Merck & Co., Whitehouse Station, New Jersey). Voluntary recall in the United States for certain lots due to lack of sterility assurance; testing identified the presence of *Bacillus cereus*.
- January 2007: Platelets Pheresis, Leukocytes Reduced, Irradiated (Department of the Air Force, 59th Medical Wing/MTLLB, Lackland AFB, Texas). Voluntary recall due to possible contamination with *Corynebacterium* species.
- December 2006: Systane® (Alcon Inc., Fort Worth, Texas) Free Liquid Gel sterile lubricant eye drops. Voluntary recall due to mold contamination.
- November 2006: COMPLETE® Moisture PLUS™ (Advanced Medical Optics Inc., Santa Ana, California) multipurpose sterile contact lens cleaning solution. Three lots sold in Japan were found to be contaminated with *Ralstonia* spp., resulting in a voluntary drug recall. Patients who used the contaminated products reported eye infection symptoms such as redness, pain, blurry vision, discharge, and swelling.
- June 2006: Comfort Shield® Perineal Care Washcloths (Sage Products Inc., Cary, Illinois) recalled owing to bacterial contamination with *Burkholderia cepacia*; the use of such contaminated product may cause serious infections including bacterial sepsis, especially in immunocompromised, and hospitalized patients.
- May 2006: ReNu® with MoistureLoc® (Bausch & Lomb, Rochester, New York) permanently withdrawn from market by manufacturer because of contamination with the mold *Fusarium keratitis*; the contact lens cleaner, supposed to be sterile, was viewed as the potential root cause of an outbreak of fungal eye infections known to cause blindness.
- April 2006: NeutraGard® (Pascal Company Inc., Bellevue, Washington) 0.05% Neutral Sodium Fluoride Anticavity Treatment Rinse and NeutraGard® (Pascal Company Inc., Bellevue, Washington) PLUS 0.2% Neutral Sodium Fluoride Anticavity Treatment Rinse, both in 16 oz bottles, recalled because of contamination with *Burkholderia cepacia* and *Pseudomonas aeruginosa*.
- August 2005: Alcohol-free mouthwash and hygiene kits containing mouthwash distributed by Medline Industries Inc., Mundelein, Illinois, recalled after being found contaminated with *Burkholderia cepacia*.
- August 2005: Trypan Blue 0.06% Ophthalmic Solution marketed as VisionBlue® (Dutch Ophthalmic USA, Kingston); this sterile product, intended for use during cataract surgery, was recalled because of contamination with *Pseudomonas aeruginosa*.
- May 2005: Gas-X with Maalox Extra Strength Antigas plus Antacid (24 count blister package) manufactured by Novartis Consumer Health Care, Carolina, Puerto Rico, recalled because of microbial contamination with *Staphylococcus aureus*.
- May 2005: Dietary Supplements Fortified Mineral Neutralizer and Ultra Fortified Mineral Neutralizer by Master's Miracle Inc., based in Minneapolis,

Minnesota, recalled because of contamination with *Pseudomonas aeruginosa*, *Pseudomonas fluorescens/putida*, and *Enterobacter cloacae*; manufacturer was promoting these products for ophthalmic use, including treatment for cataract and allergy symptoms. If used in this manner, the contaminated products could have led to serious eye injury including blindness.

- March 2004: Twice-A-Day 12-Hour Nasal Spray, manufactured by Major Pharmaceuticals, Livonia, Michigan, found to be contaminated with *Burkholderia cepacia*. A statement indicated that use of this contaminated product could cause serious or potentially life-threatening infections in patients with immunocompromised systems, particularly in individuals with cystic fibrosis.

Nonsterile Products

Microbial contamination of raw materials used to manufacture dry formulations (e.g., tablets) is often reduced by drug manufacturing processes such as granule drying and tablet compaction. The amount of bioburden reduction is directly dependent on the process temperature, chemical properties of the drug formulation, tablet compression pressure, and metabolic properties of the contaminating microbes. For example, bacterial spores are less susceptible to the harsh conditions encountered during tablet processing and the survival of *Bacillus subtilis* spores found in raw materials has been studied and documented [1]. Although dry formulations are less susceptible to microbial contamination, the spoilage of solid dosage form products by vegetative organisms has also been observed, especially in tropical and humid climates. As reported in the *Pakistan Journal of Scientific and Industrial Research* [2], various types of tablets, both coated and noncoated, were found to be contaminated with bacteria such as *Klebsiella aerogenes*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Fungi were also isolated from the samples tested and those included *Penicillium chrysogenum*, *Aspergillus flavus*, *Candida albicans,* and *Saccharomyces* spp. The total bacterial count in the contaminated products ranged from 2.0×10^3 to 8×10^7 colony forming units (CFU) per mL of sample preparation, and the total fungal count ranged from 20 to 1.4×10^2 CFU per mL of sample preparation. This article indicated that the contaminating microbes were capable of proliferating in the product by utilizing the chemicals in the drug formulations as sole carbon and energy sources.

Syrups containing a high concentration of sugar (approximately 85%) resist bacterial growth owing to the exosmotic effect on microorganisms; products containing more than 15% alcohol, such as elixirs and spirits, are usually self-preserving. However, microorganisms can survive under the harshest environmental conditions—one classic example is the contamination of various antimicrobial compounds, including povidone iodine, with *Pseudomonas* species. Therefore, the regulatory agencies expect companies to maintain strict adherence to microbial contamination control practices during the manufacture of any type of pharmaceutical drug product and to develop microbial specifications for their nonsterile products.

As a result of tightened worldwide regulatory specifications for raw materials as well as harmonization and enforcement of quality systems for nonsterile drug manufacturing, the microbial quality of nonsterile pharmaceutical products has improved over the years. However, the potential still exists for nonsterile products to become

TABLE 2.1
Antimicrobial Processes Used in Pharmaceutical Manufacturing

Type of Process	Agent	Application
Physical	Dry heat (≥160°C)	Sterilization
	Moist heat (≥121°C)	Sterilization
	Moist heat (≥100°C)	Disinfection
	Cold/freezing	Preservation
	Ultraviolet radiation	Sterilization
	Ionizing radiation	Sterilization
	Filtration (0.1–0.2 μm membranes)	Sterilization
Chemical	Ethylene oxide	Disinfection/sterilization
	Acids and esters	Disinfection/sterilization
	Alcohols, phenols, cresols	Disinfection/sterilization
	Aldehydes and aldehyde-releasing agents	Disinfection/sterilization
	Halogens	Disinfection/sterilization
	Quaternary ammonium compounds	Disinfection/sterilization
	Biguanides	Disinfection/sterilization
	Organic mercury compounds	Preservation
Combined processes	Heat + chemical	Sterilization
	Irradiation + chemical	Sterilization
	Thermoradiation	Sterilization

contaminated with types and levels of organisms exceeding product specifications since nonsterile drug manufacturing is a bioburden *controlled* process. One way to prevent or reduce microbial contamination is by introducing contamination control steps for materials and equipment at various stages of the production process. Table 2.1, adapted from Block [3], gives examples of antimicrobial treatments that apply to both sterile and nonsterile drug product manufacturing.

The majority of the contaminants of nonsterile pharmaceutical products and ingredients are bacteria, yeasts, and filamentous fungi (molds). These organisms have a wide range of nutritive requirements and environmental conditions suitable for their proliferation. Many of the ingredients used in pharmaceutical formulations can become substrates for microorganisms when the right conditions, including water activity, pH, and temperature are present. For example, most pharmaceutical preparations are within an ideal pH range (6.5–7.5) to support microbial growth, although bacteria usually favor an environment at a higher pH range and fungi favor an acidic medium. In general, low microbial levels are associated with products of low water availability, low pH, and high sucrose content. Many pharmaceutical excipients (substances other than the active drug substance or finish dosage form added to the formulation for a specific function) are low-molecular-weight compounds and therefore more prone to microbial degradation. Conversely, the active ingredients of most drug formulations are high-molecular-weight or aromatic compounds, which are more resistant to microbial degradation.

Physical changes usually seen associated with microbial contamination/product degradation are the breakdown of emulsions, the formation of pellicles, surface growth, and the production of gas, odors, or unwanted flavors. Sometimes, although no visible sign of contamination is observed, certain types of organisms may still be present in very high numbers.

Microorganisms can also cause problems even after they are dead, and therefore not detected during product testing. Their by-products and fragments may produce pyrogenic or adverse reactions in sensitized patients, resulting, for example, in food poisoning; the greater the degree of contamination, the higher the risk for toxic shock with possible fatal consequences. Sometimes, even low numbers of microorganisms that normally would not be harmful to a healthy adult could pose a health risk to a child, the elderly, or people with weak immune systems.

In order to ensure the microbial quality of raw materials, excipients and drug products, a scientifically sound quality control testing program must be implemented. Therefore, it becomes critical for microbiologists in charge of method development and validations to have a thorough understanding of the chemistry as well as the microbial and physical attributes of the material being evaluated.

Microbial Limit Standards

Most regulatory agencies and drug manufacturers have attempted to set suitable microbial limit standards for incoming raw materials and excipients used in drug product manufacturing. In limiting the microbial load of these products, one can reduce the risk for product spoilage, product recalls, and potential patient infection relative to the product's end use. Tables 2.2 and 2.3 provide an overview of standards set for some of the raw materials and excipients used in pharmaceutical formulations in the United States and Europe.

When assigning microbial limit specifications for raw materials, excipients and drug formulations, consideration is given to the process to which the product is subjected, the current technology for testing, and the availability of quality material for examination in the compendia. In the Compendia, monographs for some articles require freedom from one or more species of specified organisms. For some articles, a specific limit on the total aerobic microbial count (TAMC), total bacterial count (TBC), and/or total combined yeasts and molds count (TYMC) is given in individual monographs. The selection of limits and specified organisms to be screened for is based on the origin of the product (i.e., plant, animal, or synthetic) and intended use; for example, routine testing for the presence of *Salmonella* species in natural products of plant and animal origin is suggested. Also recommended is the testing of oral solutions and suspensions for the presence of *Escherichia coli.* Articles applied to the skin are tested for the absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Articles intended for rectal, vaginal, or urethral administration are tested for the absence of fungi. Further details on this topic are provided in Chapter 8.

THE PRESERVATION OF PHARMACEUTICAL PRODUCTS

Preservatives are chemical antimicrobial agents added to formulations to control growth and survival of microbes that may be inadvertently introduced during prod-

TABLE 2.2
USP31-NF26—Example Microbial Attributes for Raw Materials and Excipients

	Tests for Presence of					
	TAMC	TYMC	*Sta.*	*Psa.*	*E. coli*	*Salm.* spp.
Material	(NMT CFU/g or mL)		(presence/absence test)			
Acacia						X
Agar						X
Alginic acid	200 TBC				X	X
Betadex	1000 TBC				X	X
Benzalkonium chloride solution (<5.0%)				X		
Caramel					X	X
Gelatin	1000 TBC				X	X
Lactose monohydrate	100	50			X	
Mg stearate	1000	500			X	X
Sodium alginate	200 TBC				X	X
Corn starch (absorbable dusting powder)	1000	100	X	X	X	
Compressible sugar					X	X
Sugar spheres	100		X	X	X	X
Talc (topical administration)	100	50				
Xanthan gum					X	X

Note: TAMC: total aerobic microbial count; TYMC: total combined yeasts and molds count; TBC: Total bacterial count; *Sta.*: *Staphylococcus aureus*; *Psa.*: *Pseudomonas aeruginosa*; *E. coli*: *Escherichia coli*; *Salm.* spp.: *Salmonella* species; NMT: not more than.

uct use. A desirable preservative system must be free of toxic or irritant effects at the concentrations used, have adequate stability to endure heat and prolonged storage, not be adversely affected by the container/closure system, have an acceptable odor and color, and be chemically compatible with the product formulation. Some pharmaceutical formulations do not need a preservative, because the active ingredient is bactericidal and chemically interferes with reproduction of microbes, thus providing some protection against microbial proliferation. Microbial contamination that could potentially arise during product manufacture rather than during product use can be prevented by controlling people (aseptic technique and behavior), environment, and the microbial quality of raw materials and other excipients; preservatives should never be used as a substitute for cGMPs.

Antimicrobial Activity and Efficacy

The antimicrobial activity of a chemical depends on its molecular structure; for example, only the undissociated molecular form of a preservative is active because the ionized portion cannot bind to and penetrate the microorganism. In addition,

TABLE 2.3
European Pharmacopoeia (EP) 5th Edition (2005)—Example Microbial Attributes for Raw Materials and Excipients

	Tests for Presence of					
	TAMC	TYMC	*Sta.*	*Psa.*	*E. coli*	*Salm.* spp.
Material	(NMT CFU/g or mL)		(presence/absence test)			
Acacia	10^4				X	
Agar	10^3				X	X
Alginic acid	10^2				X	X
Bentonite	10^3					
Gelatin	10^3				X	X
Guar galactomannan	10^3				X	X
Lactose monohydrate	10^2				X	
Maize starch	10^3	10^2			X	
Sodium alginate	10^3				X	X
Sodium starch glycolate (types A and B)					X	X
Talc (for oral administration)	10^3	10^2				
Talc (for topical administration)	10^2	10^2				
Tragacanth	10^4				X	X
Wheat starch	10^3				X	

Note: TAMC: Total aerobic microbial count; TYMC: total combined yeasts and mold count; TBC: total bacterial count; *Sta.*: *Staphylococcus aureus*; *Psa.*: *Pseudomonas aeruginosa*; *E. coli*: *Escherichia coli*; *Salm.* spp.: *Salmonella* species; NMT: not more than.

to be effective, a sufficient concentration of the preservative must be available in the aqueous phase of the formulation because microorganisms are generally present in this product phase. Some preservatives are more soluble in oil than water, and therefore, additional amounts of the preservative must be added to the formulation to achieve adequate antimicrobial efficacy.

Antimicrobial action is also a complex process, and in many cases, a particular chemical affects more than one cell constituent and inhibits different metabolic pathways. As seen in Table 2.4, adapted from Hugo and Russell [4], some chemicals are inhibitors of bacterial cell wall and protein syntheses, whereas others interact with nucleic acids or are classified as chelating agents. One example is the effect of EDTA in chelating ions of magnesium (Mg^{++}) and calcium (Ca^{++}) in the outer membrane of Gram-negative bacteria. Although most antimicrobials can effectively intervene with the reproductive process of vegetative cells, few chemicals are sporicides. Sporulation is a complex process, and chemical agents able to affect the viability of spores are inhibitory at only certain stages in the overall life cycle of the spore former. Organisms that are injured but not killed by antimicrobials may be able to repair the cell damage and, when the opportunity is given, the microbes may proliferate and adversely affect the microbial quality and safety of the product.

TABLE 2.4
Antimicrobials and Target Cell Sites

	Cell Wall	Enzymes w/ Thiol Groups	Thiol Groups	Adenosine Triphosphate	Membrane Permeability	Coagulation	Ribosomes	Nucleic Acids	Amino Groups	Multitargeted Reactors	General Oxidation
Acridine dyes								X			
Alcohols					X						
Chlorhexidine					X	X					
Ethylene oxide		X	X	X					X	X	X
Formaldehyde	X	X	X	X	X	X	X	X	X	X	X
Glutaraldehyde	X	X	X			X			X	X	X
Hydrogen peroxide		X	X				X				
Hypochlorites, chlorine releasers	X	X	X					X	X	X	X
Iodine		X	X								
Mercurials	X						X				
Phenols	X				X	X					
Quaternary ammonium compounds					X	X					

Antimicrobial effectiveness can be impacted if the chemical agent binds with organic compounds in the drug formulation or with certain types of container/closure systems, limiting its availability to interact with viable contaminating organisms. Other types of chemicals present in the drug formulation may also impact preservative effectiveness either in a synergistic or antagonistic manner; for example, nonionic surfactants can inhibit interactions between the preservative and microbial cell membranes, whereas cationic surfactants can facilitate antimicrobial effects because bacterial cell surfaces are usually negatively charged. Physical factors, such as temperature, impact effectiveness of preservatives, and generally an increase in temperature results in increased antimicrobial activity; however, high temperatures may cause evaporation or even degradation of the preservative system. Antimicrobial efficacy can also be impaired by chemical resistance developed by certain types of microorganisms either via a natural process or acquired, in the case of mutations.

The efficacy of antimicrobial protection is demonstrated when a drug product is subjected to a compendial method such as the one described in the USP Chapter <51> *Antimicrobial Effectiveness Testing.* The compendial standard test organisms used to challenge the antimicrobial products are *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger.* Criteria for antimicrobial effectiveness are based on product category (e.g. injectables, otic, topical, nasal, oral, antacids, etc.) and type of challenge organism (bacteria or fungi). A detailed discussion on the antimicrobial effectiveness testing is outside the scope of this book. However, the methodologies used for verification of method suitability (neutralization and microbial recovery) are similar to the ones used for bioburden and microbial limit testing and those will be addressed in Chapter 7.

Types of Preservatives

Different types of preservative compounds and combinations of preservatives are used in pharmaceutical formulations. Table 2.5, adapted from Block [3], gives the usual concentration and application of some pharmaceutically useful preservative systems. A description of typical chemicals used as preservative systems in drug product formulations follow.

Alcohols

Alcohols are bactericidal against vegetative microbial cells. Ethanol is most effective at concentrations between 60–95%. When used as a preservative system, the typical concentration is 15–20%. Its use in oral preparations is useful but limited because of factors such as taste. Isopropanol has a slightly higher bactericidal effect but is more toxic. Benzyl alcohol is recommended for injectable (parenteral) products because it has bactericidal and local anesthetic properties.

Benzalkonium Chloride

Considered a first-generation quaternary ammonium compound (cationic surfactant), this antimicrobial agent is typically used in injectable and ophthalmic formulations. It has a narrow spectrum of antimicrobial activity: good activity against

TABLE 2.5
Preservative Systems Used in Pharmaceutical Formulations

Type	Typical Concentration (%)	Antimicrobial Spectrum/Application
Alcohols	15.0–20.0	Broad spectrum Ophthalmic preparations and parenterals
Benzoic acid and salts	0.05–0.1	Antifungal agent Topical and oral formulations
Benzalkonium chloride	0.004–0.02	Narrow spectrum Parenterals and ophthalmic preparations
Benzyl alcohol	0.5–5.0	Broad spectrum Ophthalmic preparations and parenterals Local anesthetic properties
Boric acid and salts	0.5–1.0	Antifungal agent Mild antiseptic products
Chlorhexidine	0.0025–0.01	Broad spectrum Dental and oral health products Ophthalmic preparations and parenterals
Cresol	0.1–0.5	Broad spectrum Limited to immunologic products
Dowicil 200	0.02–0.3	Broad spectrum Leave-on type (cosmetics and personal care products)
Parabens	0.0001–0.2	Broad spectrum and synergistic effect Liquids, emulsions, creams, and lotions
Phenol	0.2–0.5	Broad spectrum Ear and nose products, parenterals, throat lozenges, mouthwash products
Sorbic acid and salts	0.05–0.2	Antifungal Topic and oral products
Thiomersal	0.001–0.1	Broad spectrum Contact lens solutions, ophthalmic preparations, vaccines

Gram-positive bacteria at very low concentrations; requires higher concentrations to be effective against Gram-negative bacteria; and has not been shown to have any useful fungicidal or sporicidal activity. Benzalkonium chloride is water soluble and is most effective at neutral or slightly alkaline pH, becoming practically inactive below a pH of 3.5.

Benzoic Acid and Salts

Benzoic acid is used in many topical and oral formulations in its sodium salt form or in combination with other preservatives. A limitation of its use is the pH of the formulation, because the nonionized acid is the active substance. This compound

is recommended for formulations having a pH less than 4.0. Some microorganisms develop resistance to these chemicals.

Boric Acid and Salts

Boric acid and borates have been used for centuries as cleaning and preservative agents. In pharmaceutical formulations, these chemicals are not typically used as preservatives but rather as mild antiseptic products. Dilute solutions of boric acid are commonly used in eye wash formulations and other general topical cleaning/antiseptic products.

Chlorhexidine

Chlorhexidine has the greatest antibactericidal activity at a pH level of 7 to 8. It has cidal effects against Gram-positive and Gram-negative bacteria and yeasts; it also seems to have very low-level toxicity both locally and systemically and shows no permanent retention in the body. As a result, chlorhexidine is often used as an antimicrobial in dental and oral health products as well as a preservative in ophthalmic formulations.

Cresol

Cresol is a mixture of *o*-, *m*-, and *p*-methyl phenol. It has a spectrum of activity similar to that of phenol. Because of its toxicity and product incompatibilities, cresol has limited application as a preservative in pharmaceutical formulations, being used these days primarily in immunologic products.

Dowicil 200

This highly effective biocide is used mostly in cosmetics and personal care products, such as lotions, creams, baby products, and eye area products. It is compatible with most formulation ingredients and because of its great antimicrobial efficacy against pseudomonads, staphylococci, and coliforms, Dowicil 200 is a preferred preservative for leave-on types of topical products.

Mercurials

Because of their potential hazard, mercurials have declined in use over the years. Thiomersal is still used as a preservative for contact lens solutions and ophthalmic preparations, and in biological products such as vaccines. Its use is limited to alkaline and neutral formulations.

Parabens

The methyl, ethyl, propyl, and butyl esters of p-hydroxybenzoic acid are widely used in combination (usually methyl and propyl) with one another in liquid, emulsion, cream, and lotion formulations. The antimicrobial effectiveness of these chemicals is greater against Gram-positive bacteria as compared to Gram-negative bacteria. Parabens are odorless and do not cause product discoloration. Their different solu-

bility levels enable the formulator to protect effectively both the aqueous and oil phases of a formulation when the right combination of the esters is used.

Phenol

This highly effective antimicrobial agent has a broad spectrum of application. However, in the pharmaceutical industry its use as a preservative has decreased over the years because of its toxicity, odor, and incompatibility with formulation ingredients. Phenol is still used in some medicinal products such as ear and nose formulations, injectables, throat lozenges, and mouthwashes.

Sorbic Acid Salts

Sorbic acid salts are widely used in topical and oral products such as gels and syrups as fungal inhibitors. They are considered one of the least toxic preservative agents. One disadvantage is that their activity decreases with increased pH of the formulation, thus limiting their application.

Over the years, preservatives have been responsible for many drug side effects experienced by patients not caused by the active pharmaceutical ingredient (API), mainly because of allergic reactions. As a result, customers are asking for "preservative-free" products or "natural" products. One way to achieve "natural preservation" is to reduce the water activity in a product. Water activity is the amount of water that is available for use in biological processes. Water activity can be reduced, for example, through dehydration or binding of the water molecule with solutes or humectants. Products such as glycerin, alcohol, and salt can be used as biding agents. Reducing water activity does not necessarily cause reduction in moisture. Water is still in the product, but it is bound and thus not available for use. Therefore, microorganisms that might be introduced into the product will be unable to survive or multiply.

Indeed, the future of multidose drug formulation is certainly to move away from using antimicrobial chemicals as preservatives and more toward developing drugs that would result in naturally preserved products. Companies are also working on innovative approaches to package design for the production of multidose containers that can prevent the ingress of air and contamination. However, in order to truly ensure product quality and safety, microbiological control must be emphasized and enforced in all aspects of product manufacturing, from raw material procurement areas and procedures to packaging facilities. Innovations in containment technology, equipment and facility designs, air filtration systems, cleaning/disinfection products, and personnel garment designs will certainly play a critical role in the overall effort to achieve excellence in safety and microbial quality of biological, cosmetic, personal care, and pharmaceutical products.

MICROBIOLOGICAL CONTROL

Microbiological control is a regulatory requirement and one that can be defined as the continued interaction of science and applied technology with products, processes, materials, equipment, and personnel entering the manufacturing areas. In the Code of Federal Regulations (CFR) Title 21 Parts 210 and 211, control of microbial

contamination is addressed in several subparts, including Subpart C–Buildings and Facilities, section 211.42, and more specifically, in Subpart F–Production and Process Controls, section 211.13.

A good microbiological control program starts with understanding the risks for microbial contamination of the manufacturing process and identification of possible types of contaminants. The results obtained from such risk assessment can be used during facility and equipment designs as well as when establishing equipment and personnel flow patterns. Once possible sources of contamination have been identified, control and preventative measures can be implemented and qualified/validated.

Risk Assessment

The principle of risk assessment as a tool to improve pharmaceutical processes was introduced by the FDA in August 2002 with the announcement of an initiative called *Pharmaceutical cGMPs for the 21*st *Century—A Risk-Based Approach* [5]. This initiative was designed to enhance and modernize the regulation of pharmaceutical manufacturing and product quality through the implementation of process understanding principles, risk management in manufacturing, regulatory inspections, and the practice of decision making based on sound scientific principles. With this initiative, the FDA created an atmosphere for change in the pharmaceutical industry—a shift in paradigm with the focus on process understanding and implementation of new technologies.

Process risk assessment tools such as Failure Mode and Effect Analysis (FMEA) and Hazard Analysis Critical Control Point (HACCP) have been successfully used by pharmaceutical companies to identify areas in the process and types of raw materials and equipment that are at high risk of being contaminated with microorganisms [6].

HACCP, which was developed in the 1970s by the U.S. Department of Agriculture to address food safety, is *a systematic, proactive, and preventative tool to identify, assess, and prevent or reduce potential risks that can occur at specific steps in a process*. Through the risk analysis process, critical control points are identified and monitored [7]. Because microbial contamination can be introduced into the process through raw materials and excipients, clean utilities, equipment (design and flow), facilities (design, materials of construction, ventilation/air filtration systems, temperature, and humidity), and personnel, these medium-to-high-risk areas should be thoroughly evaluated and, whenever applicable, appropriate controls put into place. For example, because people are the major source of microbial contamination in a manufacturing environment, companies should focus on effective aseptic technique training and cleanroom behavior and establish personnel flows and maximum number of people for their various manufacturing suites. Some other considerations during a HACCP assessment should include

- Possibility of survival/proliferation of organisms in the product
- Potential for contaminants to produce toxins/toxic products
- Equipment-cleaning and sanitization procedures
- Facility-cleaning and sanitization procedures
- Evaluation of personnel involvement with the process
- Open versus contained/closed processes
- Processing time limits (holding times of in-process materials)

Establishing production time limits should be considered during process validation studies as a tool to control and prevent microbial proliferation in the product being manufactured. As stated in 21CFR 211.111, time limits for the completion of each phase of a production run must be established and followed, whenever appropriate. For example, if a company implements a practice to hold a bulk drug product for an extended period of time before filling into the final containers, a holding time limit must be established to prevent microbial proliferation, thus ensuring the microbial quality of the final product. Typically, as part of a product hold-time validation study, bioburden/microbial limit testing is performed at time zero and then at the end of the storage period.

Indeed, through problem-solving/risk analysis techniques and validation studies designed on the basis of sound scientific principles, pharmaceutical companies can create innovative approaches and develop effective procedures to prevent microbial contamination of drug products. Some of the critical programs at a pharmaceutical company that have a direct impact in the microbial quality of the products manufactured are

- Testing of raw materials and product samples for bioburden
- Cleaning/disinfection of facilities
- Equipment cleaning/validation studies
- Monitoring of facilities, environment, and personnel
- Microbiological testing and validation of water systems

In this chapter, the use and qualification of disinfectants will be discussed. Testing of raw materials and product samples (bioburden/microbial limit testing) will be addressed in Chapter 3; microbiological testing and validation of water systems will be addressed in Chapter 4; monitoring of facilities, environment, and personnel will be addressed in Chapter 5; and microbiological testing for equipment cleaning validation samples will be addressed in Chapter 6.

Objectionable Organisms

In pharmaceutical microbiology, there is a need and interest to screen for the presence of microorganisms that are *objectionable* to the process and products manufactured. The FDA clearly states in 21CFR, 211.113(a) that "written procedures, designed to prevent *objectionable* microorganisms in drug products not required to be sterile be established and followed." Until recently, there were four organisms of concern listed in the USP: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella* spp., and products were screened for presence of one or more of these microbial species. These microorganisms are known pathogens and, when present in a product sample, often indicate the potential presence of similar organisms of concern. However, over the years it became evident that many pharmaceutical products were contaminated with microorganisms other than the four species listed in the USP, and many of them could not be recovered using the given compendial methods used to screen for these bacterial species. Therefore, the regulatory agencies started to enforce the use of suitable methods for the screening

of microorganisms that are known to be objectionable to a particular product or process. Method suitability is indeed one of the main concerns these days in pharmaceutical microbiology because compendial methods are designed for the recovery of culturable clinical isolates and often fall short of addressing the needs for the recovery of stressed and environmental isolates. This topic is further addressed in Chapter 3.

Although the regulatory agencies expect companies to be diligent about potential objectionable microbes in their products, they do not tell a company which organisms they should screen for. Instead, the onus is on the pharmaceutical company to decide which organisms are objectionable and implement programs for product and raw material quality control testing accordingly. The decision on whether an organism is objectionable or not is up to the drug manufacturers, because the definition of objectionable is product and process dependent.

Microorganisms may be deemed objectionable based on several factors, and often the decision is made on a case-by-case basis and using a risk-based approach. Some organisms may not be pathogenic or opportunistic pathogens, but can adversely impact the quality of the product being manufactured. As a guideline, some of the factors to be taken into consideration when deciding whether a given microbe should be deemed objectionable or not include

- Type of microbial species
- Numbers of microorganisms isolated
- Product dosage form
- Intended product use
- Target patient population
- Route of administration

If the presence of a particular type of microorganism has the potential to adversely affect product quality (to include stability) and safety, then the organism should be deemed objectionable. As such, the manufacturing company should implement appropriate screening tests for raw materials and product samples. Likewise, microorganisms that have the potential to adversely affect the integrity of the product container closure system (e.g., fermenting organisms that create gaseous pressures) and/or the bioavailability of the API should also be deemed objectionable.

Most microorganisms found in a pharmaceutical manufacturing environment are Gram-positive bacilli and Gram-positive cocci (generally human-borne types), yeasts, and filamentous fungi (mold); many of these types of isolates are nonpathogenic and therefore not considered objectionable. However, some of these microbes, such as *Staphylococcus epidermidis*, can become opportunistic pathogens for patients with weak immune systems. Gram-negative organisms are generally found in aqueous environments (e.g. water systems) and raw materials of natural origin. These types of organisms are usually pathogenic and produce toxins such as endotoxins (lipopolysaccharides in the cell wall of Gram-negative bacteria). Bacterial endotoxins cause pyrogenic (fever) reactions. Therefore, products with direct contact with the blood stream (e.g., injectables, wound ophthalmics and topicals) should have specifications for bacterial endotoxin concentration.

The FDA Center for Food Safety and Applied Nutrition (CFSAN) has published a handbook on food-borne pathogenic microorganisms (bacteria, viruses, and parasites) and natural toxins referred to as the "Bad Bug Book," which can be used as a guide for the pharmaceutical microbiologist. Listed in this handbook are the following known pathogenic bacteria: *Salmonella* spp., *Clostridium botulinum*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Yersinia enterolytica* and *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, *Vibrio cholerae* OI, *Vibrio vulnificus*, *Clostridium perfringens*, *Bacillus cereus*, *Aeromonas hydrophila* and other species, *Plesiomonas shigelloides, Shigella* spp., and *Streptococcus* spp. Also included, the enterovirulent *Escherichia coli* (Eec) group that includes *Escherichia coli*—enterotoxigenic (ETEC), *Escherichia coli*—enteropathogenic (EPEC), *Escherichia coli* O157:H7 enterohemorrhagic (EHEC), and *Escherichia coli*—enteroinvasive (EIEC). Other organisms that have also been found responsible for human disease and infection include *Aeromonas* spp., *Arcanobacterium haemolyticum*, *Aspergillus* spp., *Bacillus* spp., *Bacteroides fragilis*, *Bordetella pertussis, Burkholderia cepacia, Candida albicans* and other species, *Chlamydia pneumoniae, Corynebacterium diphtheria, Cryptococcus* spp., *Enterococcus* spp., *Helicobacter pylori*, *Klebsiella pneumoniae, Microsporum* spp., *Moraxella catarrhalis, Mycobacterium turbeculosis*, *Mycoplasma pneumoniae, Neisseria meningitidis, Neisseria gonorrhoeae, Nocardia* spp., *Proteus mirabilis, Pseudomonas* spp., *Serratia marcescens, Staphylococcus epidermidis*, and *Trychophyton* spp. [8]. It seems that with the increase in the number of people with weak immune systems and microorganisms that have developed resistance to antimicrobials, the list of organisms of concern continues to grow. Therefore, it behooves the pharmaceutical microbiologist to stay abreast of the latest publications on the topic of objectionable organisms, to include published product recalls listed in trade publications such as *The Gold Sheet*, and in the FDA Web site.

Sanitization and Disinfection Practices

Sanitization and disinfection practices should be part of a microbial control program. Pharmaceutical products are at risk of microbial contamination during the manufacturing process, and therefore, procedures must be in place to ensure the microbial quality of the manufacturing environment. The USP Chapter <1072>, *Disinfectants and Antiseptics*, is a useful guide to pharmaceutical companies because it provides information on the selection of chemical disinfectants and antiseptics and the demonstration of their antimicrobial efficacy (disinfectant qualification studies). Chapter <1072> also addresses the application of disinfectants in sterile pharmaceutical manufacturing as well as regulation and safety considerations. The USP states that biofilm formation and its relationship to disinfectants are outside the scope of Chapter <1072>, a topic that will be addressed in Chapter 10 of this book in detail.

The control of microbial contamination at manufacturing facilities is addressed via a company's cleaning and disinfection procedures, which include physical and chemical methods for removal and destruction of microbes. Physical means of cleaning are capable of removing particles and debris that can harbor microbial cells.

Chemical sanitizers and disinfectants reduce microbial contamination by inactivating microorganisms that might be present in the environment.

Chemical agents that destroy microorganisms are classified as *sanitizers*, *disinfectants*, and *sporicides*. Products that kill only bacteria are referred to as *bactericidal*, whereas products that kill only fungi are referred to as *fungicidal*. Chemical agents that do not completely kill the microbes and only inhibit their proliferation are referred to as *static* agents; chemicals that inhibit bacterial growth are referred to as *bacteriostatic,* and products that inhibit fungal growth are referred to as *fungistatic*. Chemical products that are capable of inactivating all types of microorganisms, including bacterial spores, are referred to as *sporicides* or chemical sterilants. In this chapter, the use and qualification of disinfectants and sporicides as they apply to contamination control of pharmaceutical-manufacturing facilities and equipment are discussed.

Definitions and Types of Chemical Products

Sanitizers are chemical agents capable of reducing the number of viable bacteria by 99.999% in 30 s under specific test conditions [3]. These types of products have limited antimicrobial activity and are unable to inactivate bacterial spores. Sanitizers also cannot handle soil and should therefore be applied to precleaned surfaces. Examples of typical sanitizers used in the pharmaceutical industry are 70% isopropyl ethanol (IPA) and 70% ethanol. Given the fact that alcohols do not leave residue, these chemicals are widely used for sanitization of product contact and work surfaces despite their limited antimicrobial properties.

Disinfectants are chemical agents that kill vegetative forms of infectious bacteria. These chemicals have greater antimicrobial efficacy in comparison to sanitizers. Disinfectants are able to achieve 100% reduction in the number of microbial contaminants as estimated by the AOAC International 10-min Use-Dilution test, with the exception of bacterial spores and filamentous fungi [3]. Unlike sanitizers, most disinfectants available on the market are capable of handling soil, and therefore, they do not have to be applied only to precleaned surfaces. Typical disinfectant chemicals used in the pharmaceutical industry for cleaning of facilities include phenolic-based compounds, quaternary ammonium compounds, sodium hypochlorite, aldehydes, peracetic acid, and hydrogen peroxide. The antimicrobial efficacy of these compounds and the level of residue that is left on surfaces after application vary. Antimicrobial effectiveness is dependent on chemical formulation, concentration, and use; some of these products can achieve reduction of filamentous fungi (mold) and bacterial spores. Although most disinfectants and their residues are somewhat corrosive, the presence of disinfectant residues on surfaces provide for continued antimicrobial protection. Therefore, disinfectant manufacturers recommend rinsing chemical residues only in cases where significant buildup becomes an issue or to prevent corrosion of certain types of surfaces; understanding the chemistry of the disinfectants is critical so one can best recommend their use and application.

Sporicides are chemical compounds that are capable of destroying all types of organisms, including bacterial spores [3]. Because bacterial spores are more resis-

tant than vegetative cells, a sporicidal agent is considered a sterilant. These types of products are extremely corrosive to stainless steel, plastic, and soft metals and can be a health hazard to operators. It is common practice to remove chemical residues with sterile 70% IPA to reduce or prevent corrosion. Most sporicidal agents are also not capable of handling soil, and thus, must be applied to precleaned surfaces. Example of liquid sporicides widely used in the pharmaceutical industry include hydrogen peroxide–peracetic acid blends such as Spor-Klenz® (STERIS Corporation, www.steris.com), acidified (pH 5–6) sodium hypochlorite (bleach) solutions (typical concentration: 1000–2000 ppm), and Exspor® (Alcide Corporation, www.alcide.com) 4:1:1-Base (1.52% sodium chlorite). Although sporicidal agents provide for the greatest reduction in microbial contamination, their use is selective, limited to remediation events, and as an alternate product as part of a disinfectant rotation program. Other products such as chlorine dioxide, formaldehyde, peracetic acid, and hydrogen peroxide, all in gaseous form, have been used in the pharmaceutical industry as fogging agents for decontamination of closed environments (e.g., manufacturing suites and isolator systems). The CLARUS® (Bioquell Inc., www.bioquell.com) Hydrogen Peroxide Vapor Technology and the VHP® (STERIS Corporation, www.steris.com) 1000ED Biodecontamination System are examples of chemical sterilizing systems widely used for decontamination of isolators, workstations, filling lines, and rooms. These systems provide for rapid antimicrobial activity without leaving residue.

Factors in Choice and Use of Disinfectants

Careful attention must be given to the selection, preparation, storage, and application of disinfectants to ensure maximum efficacy. Most disinfectant products are produced as liquid concentrates that must be diluted with water before use. Therefore, their preparation and storage are critical to ensure the quality of the disinfectant solution that will be applied to surfaces. There are several types of products on the market, and the user must carefully evaluate which disinfectants would best suit their facility sanitization/cleaning needs. Some products may not be compatible with one another and therefore would not be suitable for rotation; others may not be compatible with the surfaces that need to be decontaminated; certain chemicals may not be allowed in certain states or countries; and some products may not be the right formulation for the application. The following is a list of key factors that should be considered when choosing a disinfectant:

- Compatibility of surfaces with disinfectant
- Operator safety
- Need for residual antimicrobial activity
- Population and types of microbial contaminants that must be eradicated
- Chemical quality, sterility, and stability
- Cleaning ability of the disinfectant
- Vendor support
- Cost and availability
- Federal and state regulations

Other factors that must be considered to ensure maximum effectiveness in the control of microbial contamination include

- Vendor's directions must be followed as written. No deviations!
- Grade of water to prepare dilutions should be process grade.
- Disinfectant dilutions must be kept in clean containers and stored for defined and qualified periods of time.
- Products used in Grade A and B areas should be sterile [9,10].
- pH of disinfectant solution should be monitored since antimicrobial activity is dependent on and affected by pH.
- Contact time of disinfectant with the surface to be decontaminated is critical and should be confirmed during disinfectant qualifications studies.
- Temperature of the diluent used in the preparation of the disinfectant solution and ambient temperature of area to be decontaminated can affect disinfectant performance. Antimicrobial activity of the disinfectant is often slower when applied to cold rooms; therefore, a longer contact time may be required.
- Organic matter present on surfaces may diminish the antimicrobial effectiveness of certain chemicals not designed to handle soil.
- Disinfectant residue should be removed only to control buildup and prevent/reduce corrosion.

Rotation of Disinfectants

The practice of rotating disinfectants as a means of proactively eradicating a broad spectrum of microorganisms that may be present in a facility is nowadays common practice in the pharmaceutical industry as well as a regulatory expectation. In the "EC Guide to Good Manufacturing Practice, Revision to Annex 1" [9], it is stated that "where disinfectants are used, more than one type should be employed. Monitoring should be undertaken regularly in order to detect the development of resistant strains." The practice of rotation of disinfectants is also mentioned in the FDA guide for aseptic processing [10] and in the USP Chapter <1072>.

Rotation of disinfectants has been and continues to be a highly debated topic with many experts on opposite sides of the debate; some state that they have scientific data to prove the need for rotation, whereas others back up their justification for *not rotating* with more data. One fact that is easy for all to agree on is the real and current regulatory expectation and enforcement of rotation of disinfectants. However, regulators do not stipulate the types of disinfectants to be used or the frequency with which these chemicals should be used. It is up to each company to evaluate its manufacturing facilities and depending on trended environmental monitoring data, establish a sound cleaning/disinfection program that should include the alternate use of chemical agents with varying and broad spectrum of antimicrobial activity.

Qualification of Disinfectants

Besides rotation, there are other regulatory expectations regarding the use of disinfectants that should be considered by the user; these include *vendor qualification*, *proce-*

dures for product acceptance and rejection, and *in-house disinfectant qualification* and *disinfectant requalification studies*. Disinfectants marketed in the United States are regulated by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and administered by the U.S. Environmental Protection Agency (EPA). Chemical sterilizers that are intended for use on critical or semicritical medical devices are regulated by the FDA. According to these regulations, disinfectant manufacturers are required to supply information on product use dilution, types of microorganisms killed, and contact times required. Disinfectant manufacturers must also ensure their products meet established standards for efficacy and safety. Efficacy of disinfectants is verified by the vendor according to test methods established by the AOAC. As a user, a company is expected to perform additional studies to evaluate the effectiveness of the disinfectants as they are prepared and used and to evaluate the storage conditions of disinfectant dilutions for possible loss of efficacy over time. These studies are important because, as explained earlier in this chapter, the efficacy of a disinfectant can be affected by types of surfaces, contact time, method of application, and type of microbial flora present. In fact, users are expected to challenge disinfectants not only with standard test organisms but also with facility environmental isolates because commercially available microorganisms behave quite differently from their "wild" counterparts. Selection of the test organisms is crucial and an important issue to the regulatory agencies, especially when it comes to environmental isolates.

The test protocol used in a disinfectant qualification study varies from company to company because methods must be customized to reflect the types of surfaces and application of the chemical products at a particular facility. In general, two types of tests are performed: in situ and *in vitro testing*. The following FDA observation, published in the *BioQuality* Vol. 11(5), 2005, is a good example of the current regulatory climate in terms of disinfectant testing:

"Disinfectant effectiveness studies are inadequate in that

- Only in vitro studies were conducted.
- No product contact surface or equipment studies have been conducted.
- Organisms used in the in vitro studies failed to include yeast and/or mold.
- No expiration dating studies for opened disinfectants."

In this chapter, the current industry practices and regulatory expectations in terms of disinfectant qualification protocols are discussed.

In Situ Testing

In situ testing is performed to evaluate the effectiveness of the cleaning/disinfection procedures. Typically, these studies are carried out by monitoring the manufacturing facilities before and after routine cleaning takes place and over several days (a minimum of three consecutive days is recommended). Monitoring at worst-case conditions (e.g., after a shutdown), when there is the potential for greater number and types of microbes in the environment, provides for better assessment of the disinfection efficacy. During these studies, the number of sample sites (surface and air samples) is increased and the types of activities in the area are documented. The EM

data collected before and after cleaning are compared—a typical acceptance criterion for in situ studies requires EM data after cleaning to be below the established alert levels for the given area. In situ studies add value to the overall assessment of cleaning/disinfection effectiveness by providing real-world results from the proper use and application techniques of the chemical agents and personnel practices. However, a disinfectant qualification study is not complete unless in vitro studies are also conducted to confirm the actual antimicrobial effectiveness of the chemical agent against selected microorganisms.

In Vitro Testing

There are three main types of studies performed in this category, and all are carried out in a laboratory setting because the test methods call for challenging the chemical agents with live cultures: the AOAC Hard Surface Carrier Test Method [11], Surface Challenge Tests [12], and Use-Dilution Tests [11]. Typical challenge organisms used for in vitro studies are presented in Table 2.6.

AOAC Hard Surface Carrier Test

The AOAC hard surface carrier test is a qualitative method. The general study outline involves the use of stainless steel penicylinders inoculated with the test organisms (target load per dried carrier: $0.5–2.0 \times 10^6$ CFU) and placed into test tubes containing the disinfectant solution to be evaluated. The contaminated carriers remain in contact with the disinfectant for a specified amount of time (contact time). Following the desired contact time, the carriers are removed, placed into a neutralizing medium, and incubated at specified conditions. Following incubation, the test tubes are observed for growth. Verification of identity of the challenge organism recovered in the tubes with microbial growth is recommended. A standard performance evaluation at 95% confidence level for disinfectant activity is obtained by demonstrating kill of 59/60 replicates for each organism. A product may be classified as a sporicidal agent if kill of 60/60 replicates for each spore-forming bacteria tested is obtained. This AOAC method meets FDA and EPA criteria for disinfectant claims and is generally carried out by disinfectant manufacturers. As a user, a company may elect not to perform this test in favor of the surface test or use-dilution test, because the carrier test is very time consuming and requires skilled technicians with excellent aseptic and microbial manipulation techniques. In fact, most pharmaceutical companies no longer perform this test, which is not seen as a true requirement to demonstrate disinfectant efficacy at the user site. In the USP Chapter <1072> it is stated that in order to demonstrate efficacy of a disinfectant within a pharmaceutical-manufacturing environment, it may be deemed necessary to conduct use-dilution tests, surface challenge tests, and in situ studies.

Use-Dilution Test

The use-dilution test, also referred to as a time-kill study, is derived from the AOAC use-dilution method. This test is quantitative and demonstrates log reduction of a wide range of test organisms (to include environmental isolates) upon exposure to various disinfectant concentrations and at various contact use dilution times. The study outline typically involves inoculating an aliquot of the disinfectant with the test organism to achieve a concentration of approximately 10^6 CFU/mL. At selected

TABLE 2.6
Typical Test Organisms Used in Disinfectant Qualification Studies

Standard Challenge Organisms	Typical Environmental Isolates
Vegetative Bacteria	
Escherichia coli	*Micrococcus luteus*
ATCC 11229 (AOAC)	*Staphylococcus epidermidis*
ATCC 8739 (USP)	*Corynebacterium jeikeium*
Staphylococcus aureus	*Pseudomonas vesicularis*
ATCC 6538 (AOAC and USP)	*Rhodococcus globerulus*
Pseudomonas aeruginosa	*Pseudomonas fluorescens/putida*
ATCC 15442 (AOAC)	*Burkholderia cepacia*
ATCC 9027 (USP)	*Ralstonia pickettii*
Spore-Forming Bacteria	
Bacillus subtilis (spores)	*Bacillus sphaericus*
ATCC 19659 (AOAC)	*Bacillus cereus/thuringiensis*
ATCC 6633 (USP)	*Paenibacillus* spp.
Fungi	
Candida albicans	*Penicillium chrysogenum*
ATCC 10231 (AOAC and USP)	*Aspergillus niger*
ATCC 2091 (AOAC)	*Alternaria* spp.
Penicillium chrysogenum	*Fusarium* spp.
ATCC 11709 (AOAC)	*Paecilomyces* spp.
Aspergillus niger	
ATCC 16404 (AOAC and USP)	

ATCC — American type culture collection.

time points (generally zero, 5 min, and 10 min), aliquots of the inoculated disinfectant are removed, placed into a neutralizing medium, and selected dilutions plated (using pour-plate method or membrane filtration method) with an agar medium for microbial enumeration purposes. Prepared plates are incubated at specified conditions appropriate for the test organism being evaluated. After incubation, recovered colonies are enumerated and log reductions are calculated based on the initial inoculum. Verification of identity of the challenge organism is recommended. There is no standard test performance evaluation; however, most protocols designed by pharmaceutical companies require a minimum of 4-log reduction of vegetative bacteria and fungi in order to demonstrate disinfectant properties and a minimum of 6-log reduction in bacterial spores to demonstrate sporicidal activity.

Surface Challenge Tests

Surface challenge tests are customized procedures based on the AOAC method for germicidal spray products and designed to evaluate the effectiveness of a disinfectant

against standard and environmental isolates when applied to representative surfaces found in a manufacturing facility. This test has become the preferred disinfectant qualification method by the regulatory agencies. It is quantitative and demonstrates log reduction of the test organism upon exposure to the selected disinfectant concentration as it is used by a company during a cleaning procedure. In order to not deliberately contaminate the manufacturing areas, surface challenge tests are performed in a laboratory setting and using representative surfaces (referred to as coupons) that are scaled down to a size of about 2 × 2 in. Coupons used in surface challenge tests are often made from materials such as stainless steel, glass, vinyl, polycarbonate, Plexiglass, epoxy-coated gypsum, and terrazzo tiles.

The typical study outline involves inoculating each of the test coupons with about 0.1 mL of an inoculum suspension of vegetative cells or spore suspension (target: 10^6–10^7 CFU/0.1 mL). The inoculum is then spread evenly over the coupon and, depending on the test method, it is dried onto the test coupon. Drying often results in loss of cell viability for vegetative organisms. Therefore, most companies either use a drying step only for bacterial spores or use a higher starting inoculum for vegetative organisms to account for loss in cell viability after drying. After inoculation (and drying, if applicable), the chosen disinfectant solution is applied to the coupon surface and allowed to remain in contact with the test organisms for a specified amount of time (typical time points are zero, 5 min, and 10 min). The method of disinfectant application may vary depending on actual use at the manufacturing site; for example, the disinfectant application may include spraying or wiping with a sterile wipe saturated with the disinfectant. It is important that the test protocol be designed to best mimic the company's cleaning procedure for the type of surface being evaluated. Following the desired contact time, the treated coupons are sampled for recovery of surviving organisms using swabs, rinse, or contact plates. Neutralizers that inactivate the disinfectants should be included in the diluent (swab and rinse method) and microbiological media to ensure adequate recovery of viable cells. The aliquots of the test diluent that are plated with microbiological media or the contact plates are then incubated at specified conditions appropriate for the test organism being evaluated. At the end of incubation, the colonies recovered are enumerated and compared to untreated inoculated coupons that have been extracted in the same manner (positive controls) and the log reduction in microbial population determined. Verification of identity of the challenge organism is recommended. The standard performance evaluation for a surface challenge test is a minimum of 2-log reduction for bacterial spores and a 3-log reduction for vegetative organisms (bacteria and fungi) during the predetermined contact time [12].

Expiration Date for Disinfectant Solutions

Companies must qualify the expiration dates for prepared disinfectant solutions if they are not to be used on the day of preparation. These studies are performed to demonstrate that the diluted chemical agent will remain stable and active during the storage period and in the chosen container. The typical protocol outline for this type of study involves performing a use-dilution test on the day the disinfectant solution is prepared and once again at the end of the proposed expiry date. If there is a sig-

nificant loss of efficacy compared to the initial result, i.e., greater than a 0.3–0.5 log variation, which is defined as normal plating variability [13], the company should not store the disinfectant for the proposed time frame and should consider a shorter storage period or an alternate storage container type.

Sanitizers Used for Equipment Cleaning

Pharmaceutical-manufacturing equipment that cannot undergo steam-in-place (SIP) procedures, or autoclaving must be chemically cleaned prior to use. Chemical sanitization can be accomplished using caustic, acidic, and oxidizing agents, such as hydrogen peroxide and sodium hypochlorite solutions. Chemical sanitization of equipment using cleaning-in-place (CIP) procedures provide for automatic cleaning and disinfecting of equipment without major disassembly and assembly of parts. A CIP cycle is more of a design method than a cleaning process, and it is achieved by placing pipes at an angle to the horizontal (minimum 3%) to improve drainage, and using instruments and valves that connect flush to pipes, thereby eliminating dead legs to improve draining and prevent stagnant liquid. In order to confirm the effectiveness of the chemical sanitization procedures, companies must perform studies that are similar to the use-dilution/time kill protocols performed for disinfectant qualification testing.

A typical study outline involves inoculating separate aliquots of the chemical sanitizer with various standard test organisms, including environmental isolates, to achieve a concentration of approximately 10^6 CFU/mL. At selected time points, depending on the equipment cleaning protocol, aliquots of the inoculated sanitizer are removed, placed into a neutralizing medium, and selected dilutions plated (using the pour-plate method or membrane filtration method) with an agar medium for microbial enumeration purposes. Prepared plates are incubated at specified conditions appropriate for the test organism being evaluated. After incubation, recovered colonies are enumerated and log reductions are calculated based on the initial inoculum. Verification of identity of the challenge organism is recommended. There is no standard test performance evaluation. Most protocols for evaluation of microbiocidal properties are based on a minimum requirement of a 2-log reduction in spore-forming bacteria and a minimum 3-log reduction in vegetative bacteria [12].

As with disinfectant qualification testing, traditional test protocols for evaluating antimicrobial effectiveness of sanitizing and disinfectant solutions are established using ideal laboratory conditions with liquid cultures of free cells (planktonic cells). This allows for excellent and uniform physical contact of the antimicrobial agent and the microbial cells that are metabolically active. In cases where equipment are operated at different temperatures or there is indication of biofilm formation in the equipment, the test results obtained from these traditional qualification studies become questionable. This topic is discussed in detail in Chapter 10.

Neutralization and Microbial Recovery Studies

In order to ensure the validity of the data obtained from in vitro disinfectant qualification testing, the study protocol must include *neutralization* and *microbial recovery* studies as test controls. Neutralization studies must be performed for each type

TABLE 2.7
Neutralizing Agents for Common Antimicrobials

Antimicrobial Compound	Potential Neutralizing Agent
Alcohols	Dilution or polysorbate 80
Aldehydes	Dilution or thiosulfate
Bis-biguanide	Lecithin
Chlorhexidine	Polysorbate 80 and lecithin
EDTA	Mg^{++} and Ca^{++} ions
Glutaraldehyde	Glycine and sodium bisulfite
Halogens	Thiosulfate
Iodine	Polysorbate
Mercuric chloride and other mercurials	Thioglycollate; thiosulfate
Parabens	Polysorbate 80 and lecithin
Phenolic compounds	Dilution or polysorbate 80 and lecithin
Quaternary ammonium compounds	Polysorbate 80 and lecithin
Sodium hypochlorite	Sodium thiosulfate
Sorbates	Dilution

of organism, disinfectant, and coupon (for surface test only) combination test to demonstrate the ability of the medium to support growth of any viable organism. Table 2.7 provides a list of typical chemical neutralizers that can be used in these studies. Some culture broths are formulated with a variety of neutralizing agents and are thus considered "universal neutralizing media." Dey/Engley (D/E) is an example of a universal neutralizing broth widely used in disinfectant qualification studies. *Microbial recovery* studies are performed for each type of organism, disinfectant, and coupon (for surface test only) combination test to ensure the extraction/recovery efficiency for the viable test organisms from the test surfaces/liquid medium (test-positive controls). Neutralization and microbial recovery studies are designed based on compendial methods for validation of microbial recovery as described in the USP Chapter <1227> *Validation of Microbial Recovery from Pharmaceutical Articles,* which will be discussed in Chapter 7.

Requalification and Change Control

Requalification of disinfectants and cleaning procedures is not required unless a significant change has been made to the program. The regulatory expectation is that facilities, systems, equipment, programs, and processes (to include cleaning and disinfection) should be periodically reviewed to confirm that they remain valid and in a state of control [14]. If a significant change has been made, a company should evaluate it via a change control program. For example, if a company decides to change the types or manufacturers of qualified disinfectants/sanitizers, the proposed change must be first evaluated via a formal documentation system; sometimes, an annual review of the environmental monitoring data indicates an adverse trend or presence

of atypical organisms. In such cases, limited requalification of cleaning/disinfectant procedures may be required using the environmental strains that have demonstrated resistance to the existing cleaning procedures. In summary, periodic requalification/revalidation of approved procedures is not required as long as a company has routine monitoring and verification programs that are capable of detecting adverse trends and abnormal conditions.

CONCLUSION

Microbial contamination cost companies thousands to millions of dollars annually in equipment damage, production downtime, product contamination, investigations, and energy losses. Recently, there has been a paradigm shift in the pharmaceutical and biotechnology industries to move away from the conventional approach of dealing with microbial contamination in reactive mode to a proactive approach that includes process understanding and quality by design. Companies now seek to understand the sources of contaminants, environmental conditions, and facility and equipment designs that can lead to microbial colonization and proliferation. In addition, companies have started to evaluate alternate strategies for sanitization and cleaning verification to ensure that sanitization and disinfection procedures are effective. A better understanding of how microorganisms survive and proliferate in the manufacturing environments is also needed so that companies can implement effective microbial control strategies.

Microbial contamination control is not simply a task! It is a continuous effort involving all parts of the facility, all aspects of the process, and company personnel. It is indeed a true continuous improvement activity, one that requires the support of company management and that must be embraced by manufacturing operators involved in the production of pharmaceutical drug products.

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3 The USP Microbial Limit Tests

The methodologies described in the compendia for examination of microbial quality of nonsterile products include quantitative (bioburden) and qualitative tests for specified organisms. Since the publication of the first edition of this book, the microbial limit tests in the USP have been harmonized with the tests for microbial contamination listed in the EP and the JP. The intent of harmonization was to facilitate achievement of a single specification for a given pharmaceutical product, which would be applicable in the United States, Europe, and Japan. The EP was appointed by the Pharmaceutical Discussion Group (PDG) as the coordinating pharmacopeia for harmonization of the microbial limit tests. The revision of the USP Chapter <61>, Microbial Limit Tests, which started in 1999, has undergone numerous changes based on comments received from the industry as well as input from the subject matter experts from the three pharmacopeias. As a result of the harmonization efforts, USP Chapter <61> was renamed to reflect only microbial enumeration tests, and a new Chapter <62> was created to provide tests for the screening of specified organisms. In essence, Chapter <61> was split into two to better resemble the testing design in the EP. The new harmonized USP chapters are

- Chapter <61>, Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests
- Chapter <62>, Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms

HISTORY OF THE REVISION AND HARMONIZATION PROCESS

The USP informational Chapter <1196>, Pharmacopeial Harmonization, provides information about the concept of harmonization by the PDG, which was formed in 1989. The PDG has representatives from the European Directorate for the Quality of Medicines in the European Council, the United States Pharmacopeial Convention, and the Japanese Pharmacopoeia in the Ministry of Health, Labor, and Welfare (MHLW), and it often meets in conjunction with the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. The goal of harmonization is to reduce the burden on the part of pharmaceutical manufacturers of performing a test in different ways and using

different acceptance criteria to prove that a particular product complies with specifications for a given quality attribute. The compendial tests for microbial attributes of nonsterile products fall in this category and needed harmonization so that users could achieve conformity to specifications while performing a single test.

The original proposal for the revision of USP Chapter <61> appeared in the Pharmacopeial Forum (PF) Vol. 25(2) [Mar.–Apr. 1999] under Pharmacopeial Previews. The proposals were further revised and forwarded to In-Process Revision as Official Inquiry Stage 4 drafts published in the PF Vol. 27(2) [Mar.–Apr. 2001]. The new chapters were targeted to become official in the USP 25. However, additional changes were made, and the two revised chapters were once again published in the PF Vol. 29(5) [Sept.–Oct. 2003] as Stage 4 draft Official Inquiry documents. Since 2003, these two chapters continued to be evaluated as they navigated through the pharmacopeial harmonization process, moving from Stage 5 (consensus) to Stage 6 (adoption) in 2006. Currently, both chapters are available in the compendia with a proposed implementation date of May 1, 2009. Originally scheduled to be implemented on August 1, 2007, the date of implementation was postponed in response to many requests from users to allow sufficient time for method validation work prior to method adoption. The USP emphasizes that adoption of revised methods prior to the official implementation date is at the discretion of each user and that the decision may be subject to regulatory consideration.

In Europe, implementation of the harmonized chapters by the EP is under a different schedule as follows:

1. For pharmaceutical products and substances for pharmaceutical use that are covered by a compendial monograph specification, the existing methods can be used until the monograph is revised and implemented. It is expected that all applicable monographs will be revised by January 1, 2009. From that date on, only the harmonized methods should be used.
2. For new applications for marketing authorization not covered by a compendial monograph, the company may use either the existing method or the harmonized method until December 31, 2008. However, the use of harmonized methods is highly recommended. Starting January 1, 2009, only the harmonized methods should be used as reference documents.
3. For already approved products not covered by a monograph, the company may use either the existing method or the harmonized method until December 31, 2008. From January 1, 2009, only the harmonized methods should be used as reference documents.

USP CHAPTER <61> MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

This chapter, which has been harmonized with EP section 2.6.12 and JP section 35, provides tests for the quantitative estimation of aerobic mesophilic bacteria and fungi that might be present in pharmaceutical articles of all kinds, from raw materials to finished products. These tests are designed primarily to verify whether a sample complies with the established specification for microbial quality. In this revised chapter, the USP clarifies that these methods are not applicable to products contain-

ing viable organisms, as in the case of fermentation broths. Therefore, if there is a need to test for nonhost contamination in fermentation cultures, alternate methods should be employed. In general, alternate methods, to include automated methods, may be used to substitute the compendial tests as long as suitable method qualification studies are carried out to demonstrate method equivalency. Sample preparations may also be modified, as needed, based on the results obtained from the qualification tests (suitability testing), and any antimicrobial property present in the product must be removed or neutralized before routine testing is conducted.

Chapter <61> contains procedures for the estimation of total aerobic microbial count (TAMC) and total combined yeasts and molds count (TYMC). Most of the changes included in this chapter were needed clarification or allowance for some test flexibility. For example, provisions are made for testing less than 10 g or 10 mL of material, and incubation periods are now expressed in terms of days instead of hours. One word of caution: although incubation times are specified in terms of days, the expectation is that samples incubate for the total number of hours equivalent to the number of days specified in the method. For example, if the method states to incubate samples for 2 d, the time of incubation documented should be a minimum of 48 h.

In an attempt to harmonize with the EP, the revised USP chapter contains a section for testing of transdermal patches and a description of the spread-plate technique. The harmonized chapter also provides much more detailed information on requirements to demonstrate method suitability (method validation) and growth promotion properties of recovery media, topics addressed in Chapter 7 of this book. Traditionally, acceptable microbial variability has been defined as 0.5 log variation in microbial counts. However, in the harmonized Chapter <61>, the definition has been changed to a factor of 2 (0.3 log). A summary of the main differences between the old USP microbial limit tests for enumeration of microorganisms and the new harmonized methods is presented in Appendix A at the end of this chapter.

Sample Preparation

According to the USP, samples are prepared using a method that has been shown to be suitable for the type of product to be tested. Testing for method suitability ensures that the sample preparation will not alter any inherent bioburden present in the sample and that any antimicrobial or inhibitory properties are eliminated adequately prior to testing.

Unless otherwise specified in monographs, sample amounts equal to 10 g or 10 mL should be used for microbial limit testing. The USP also specifies that, for fluids or solids in aerosol form and for transdermal patches, a total of ten product units must be tested. However, the USP did realize that, in some cases, only a limited amount of the product will be available for testing. Hence, in the revised microbial limit chapters, there are provisions to test smaller amounts of samples, especially when the batch size is very small.

When preparing a sample for microbial limit testing, all steps must be performed using aseptic techniques and sterile materials. The USP provides recommendations for dissolving or suspending the product so that a homogeneous sample is achieved. In general, the sample preparation step involves dissolving or suspending 10 mL or 10 g of the test specimen in a benign diluent such as pH 7.0 buffered sodium

chloride–peptone solution, pH 7.2 phosphate buffer, or tryptic (trypticase) soy broth (TSB). *Note:* TSB is the same as soybean–casein digest (SCD) broth.

Usually, a 1:10 dilution is prepared; however, higher product dilutions may be needed as determined by method suitability studies. Also, if deemed necessary, the pH of the sample preparation should be adjusted to a range of 6–8 using sterile acid or alkaline solutions. When testing a solid product that does not completely dissolve in the chosen diluent, the material may be reduced to a fine powder using, for example, a sterile mortar and pestle, for better sample dispersion in the buffer solution. The USP provides guidance for sample preparation of water-immiscible products, aerosols, and transdermal patches as follows:

- Non-fatty products insoluble in water—Prepare a suspension of the product in the diluent. The use of an emulsifying agent such as polysorbate 80 at a concentration of 1 g/L of diluent is recommended to aid homogenization of the sample preparation.
- Fatty products—Dissolve the product in filter-sterilized isopropyl myristate, or homogenize it using sterile polysorbate 80 or any other suitable sterile surfactant. If needed, heat the sample preparation to not more than 40°C (or use prewarmed diluent) to help dissolve/disperse the product. In some cases, the sample preparation may be heated to not more than 45°C if proven acceptable by the method suitability studies.
- Fluids or solids in aerosol form—Aseptically transfer the product to a sterile container for further sampling. Although not specified in the USP, one may chill the container in an alcohol dry ice mixture for about 1 h. Then at room temperature and, using aseptic techniques, cut the container open and allow the propellant to escape prior to sampling the article. This type of sample preparation must not add to adventitious bioburden in the sample and must be qualified as a suitable practice for sample collection.
- Transdermal patches—remove the protective cover sheets and place the product units, with adhesive side up, in a sterile container such as a large Petri dish. Cover the adhesive side of the product units with a sterile porous material to prevent the units from sticking together. Aseptically remove each product unit and add them to the test diluent containing suitable inactivators such as lecithin and polysorbate 80. Shake the product preparation for at least 30 min, prior to collecting the sample aliquot for testing.

Total Aerobic Microbial Count

The TAMC test provides for the estimation of viable aerobic mesophilic microorganisms (bacteria and fungi) using a general-purpose medium such as SCD agar. Testing must be performed using aseptic techniques and in an environment such as a laminar flow hood or biological safety cabinet in order to prevent adventitious contamination during testing. The TAMC test applies to the testing of nonsterile samples to include raw materials, finished products, and in-process formulations. Some monographs have a test specification for total bacterial count (TBC). In this case, only bacteria recovered on the recovery medium should be enumerated and reported. The test can be performed using membrane filtration, pour-plate, spread-plate, or multiple-tube

techniques. The method chosen must reflect the type of product to be tested, must allow for testing of a sufficient amount of sample to verify compliance with product specifications, and must be validated/qualified prior to use. After samples are processed, the test plates incubate at 30–35°C for 3–5 d. At the end of incubation, recovered microbial colonies are enumerated and results are reported as number of colony-forming units (CFU) per gram or milliliter of product tested. The USP states that a suitable counting range for the TAMC test is a value below 250 CFU because overcrowding of plates can result in diminished accuracy in test results.

Total Combined Yeasts and Molds Count

The TYMC test provides for the estimation of mesophilic aerobic fungi using a general fungal medium such as sabouraud dextrose agar (SDA) or potato dextrose agar (PDA). Testing must be performed using aseptic techniques and in an environment such as a laminar flow hood or biological safety cabinet in order to prevent adventitious contamination during testing. The TYMC test applies to the testing of nonsterile samples to include raw materials, finished products, and in-process formulations. Although the TYMC test is designed to recover viable fungal microorganisms, if colonies of bacteria are detected using the fungal medium, they are to be counted as well. The test can be performed using membrane filtration, pour-plate, or spread-plate techniques. As discussed in the case of the TAMC test, the choice of method, which must be qualified, is based on the type of product to be tested and product specifications. After samples are processed, the test plates incubate at 20–25°C for 5–7 d. At the end of incubation, recovered microbial colonies are enumerated, and results are reported as number CFU per gram or milliliter of product tested. The USP states that a suitable counting range for fungi is a value below 50 CFU because overcrowding of plates, especially by mold species, can result in diminished accuracy in test results.

Bioburden Tests

A bioburden test is referred to as a total viable count (TVC) test for estimation of viable aerobic mesophilic microorganisms in products or articles not purported to be sterile. In essence, a bioburden test can be carried out as the USP TAMC test, or as the total of the TAMC and TYMC tests (TVC = TAMC + TYMC), or using a medium of choice with either single- or dual-temperature incubation conditions. In general, bioburden tests are performed for the estimation of microorganisms in samples such as containers, product contact surfaces, water, in-process samples, final bulk products prior to sterilization, and any other type of material that require assessment of their bioload, with no reference to a defined compendial requirement for sample amount. Bioburden tests are typically performed during cleaning validation studies, and the samples can be processed directly using a device containing a nutrient medium (e.g., contact plate). Alternatively, samples can be collected using swabs, swatches, or rinse fluid and then processed using the chosen microbial recovery method.

Two-Media Bioburden Test

As explained, a bioburden test can be performed as the USP TAMC and TYMC tests by the plate-count method (pour-plate or spread-plate) or membrane filtration

method. At the end of the incubation period, the counts obtained from the TAMC and TYMC tests are added and reported as the TVC. An attempt should be made to characterize colonies isolated from both media so that the same type of organism is not counted twice.

One-Medium, Dual-Temperature Incubation Bioburden Test

The one-medium, dual-temperature incubation method is designed for the detection of low bioburden of both bacteria and fungi surviving in an oligotrophic environment. Although the microbial limit tests call for the use of the sabouraud dextrose medium for the recovery of fungi, studies performed over the years have demonstrated that all-purpose media, such as SCD, are capable of recovering a wide range of bacteria, yeasts, and molds [1, 2]. The test can be performed as the USP TAMC or as a modification to the TAMC test using alternate media and alternate incubation conditions. However, a more popular approach is to modify the TAMC method and perform the test using SCD agar or microbial content test agar (MCTA), and incubate the test samples at two temperature ranges for the optimum recovery of both bacteria and fungi. This test approach is widely used in the pharmaceutical industry for microbial monitoring of environments, surfaces, and equipment, and is suggested in the Association for the Advancement of Medical Instrumentation (AAMI) guidelines for articles expected to have a low bioburden. Samples collected are typically incubated at 30–35°C for 2–3 d followed by a 5–7 d incubation period at 20–25ºC. It is recommended that plates should be observed for microbial growth at the end of the initial incubation period for detection of possible spreaders and to prevent plate overgrowth.

Variations of the dual-temperature incubation bioburden test include length of incubation of test plates as well as the order of incubation temperature range: some methods specify a low-temperature incubation period initially, followed by moderate-temperature incubation. This issue has actually been a topic of debate in the industry and among regulatory inspectors for several years. The concern was that there could be a risk of possible low recovery of fungi and certain psychrophilic organisms if plates were to be incubated initially at a moderate temperature range (30–35°C) because faster-growing mesophilic bacteria could overcrowd the plates. Nowadays, the general consensus is that the order of temperature of incubation is not a critical factor for most environmental organisms, including the typical contaminants in pharmaceutical products and facilities, especially when low-level bioburden is expected. Many studies have been performed by companies in support of a dual-temperature incubation method, and results do not show significant difference in results. In fact, most environmental fungi grow very well at 30–35°C; fastidious bacteria remain viable at 20–25°C and are readily recovered when incubated at 30–35°C. However, using an initial higher incubation temperature does have a compliance advantage: this approach is referenced in the AAMI guidelines, which, to the author's knowledge, may be the only published reference on this subject.

One point of consideration when choosing this test approach is the need to use a mixed inoculum composed of representative test organisms when performing method suitability studies. The study design must demonstrate that the various types

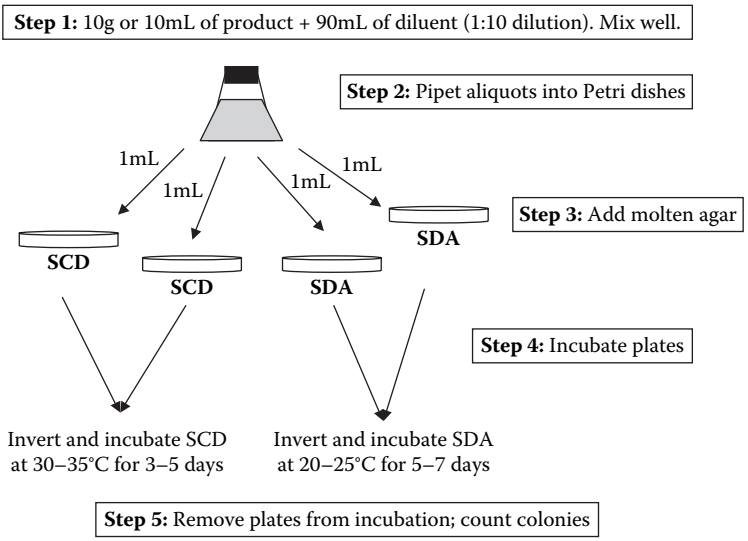


FIGURE 3.1 TAMC and TYMC tests via pour-plate method.

of challenge organisms can be recovered on the same medium without the inhibitory or masking effects of one species over another.

TAMC and TYMC Tests via Plate-Count Methods

The TAMC and TYMC tests can be performed via plate-count and membrane filtration methods. In this section, the two main techniques used in plate-count procedures will be described.

Pour-Plate Method

Typically, 10 g or 10 mL of the product is added to a diluent to make a 1:10 dilution. Then, 1-mL aliquots of the 1:10 dilution preparation are separately added to four 15 × 100 mm sterile Petri dishes. Therefore, when using this testing scheme, the test dilution factor is 10. Two of the plates receive 15–20 mL of SCD agar for the TAMC test, and the other two receive 15–20 mL of SDA for the TYMC test. The media must be cooled to approximately 45°C prior to transfer to the Petri dishes containing the sample preparation. If larger Petri dishes are used to accommodate larger sample aliquots, the volume of the molten medium added to each plate must be increased. See Figure 3.1 for an outline of the test.

Spread-Plate Method

From the sample preparation in buffer or broth diluent, transfer aliquots of not less than 0.1 mL onto two 15 × 100 mm Petri dishes containing SCD agar and two 15 × 100 mm Petri dishes containing SDA medium. (Note: for a 15 × 100 mm plate, avoid pipetting a volume greater than 0.2 mL because larger volumes make it difficult (or not suitable) to spread the sample preparation onto the agar surface.) The surface of

the agar medium should be relatively dry prior to adding the sample preparation. After adding the sample aliquot, spread the sample over the surface of the medium using a sterile spreader. When plating 0.1 mL of a 1:10 sample dilution, the test dilution factor is 100. Therefore, the spread-plate method is less sensitive compared to the pour-plate method. However, colonies are easily enumerated, and viable organisms are not at risk of suffering heat shock due to the addition of molten agar as in the case of the pour-plate method.

Incubation and Results Calculation

For both the pour-plate and spread-plate methods, SCD plates are incubated at 30–35°C for 3–5 d, and SDA plates are incubated at 20–25°C for 5–7 d. At the end of the incubation period, enumerate the recovered colonies from each agar medium. An instrument such as the Quebec® colony counter should be used for colony counting. Calculate the arithmetic mean (average) of the recovered colonies from each agar medium and report the results.

When a 1:10 sample dilution is prepared and either a 1-mL sample is tested by the pour-plate method, or a 0.1-mL sample is tested by the spread-plate method, the following statements apply:

- If counts are recovered, average the number of CFU from the duplicate plates and multiply by the dilution factor. Results are reported separately for the TAMC and TYMC tests.
- If no colonies are present, report results as < 10 CFU per gram or milliliter of product for the pour-plate method.
- If no colonies are present, report results as < 100 CFU per gram or milliliter of product for the spread-plate method.

Example 1: For a 1:10 sample dilution where 1-mL aliquots are plated, the dilution factor is 10. If 3 CFU are recovered on one SCD agar plate, and 5 CFU are recovered on the duplicate SCD agar plate, the average count is 4 CFU. Therefore, the TAMC result is 40 CFU per gram or milliliter of product tested.

The preceding example uses the USP-recommended initial product dilution (1:10) when using the pour-plate method. Many products that do not have inhibitory properties or that are relatively soluble or miscible in buffer solutions can be tested at this proposed sample dilution. However, some products, either because they are inhibitory or due to their physical properties, must be tested using higher volumes of diluent. In such cases, alternate sample dilution schemes need to be attempted and qualified as described in Chapter 7. The following are some examples of sample preparations using alternate dilution schemes and the corresponding reporting of test results.

Example 2: A 10-mL sample aliquot is diluted using 190 mL of buffer to make a 1:20 sample dilution.

2a. Duplicate 1-mL aliquots are plated with SCD agar for the TAMC test. After incubation, no colonies are recovered. Because 1-mL aliquots are plated, and results are averaged for the duplicate plates, the dilution factor is 20 [10 mL sample ÷ 200 mL total volume (1:20 dilution) × 1-mL aliquot × 20 (dilution factor) = 1] for reporting results per 1 mL basis. Therefore, for this example, the test result is reported as < 20 CFU per milliliter of product.

Dilution factor calculation:

10 mL of sample into 190 mL diluent (1:20 dilution) × 1 mL (aliquot plated) × DF (dilution factor) = 1 (result per milliliter). Therefore, DF = 20.

In order to increase the test sensitivity, 2-mL aliquots of the sample preparation can be plated instead of 1-mL aliquots. Plating duplicate 2-mL aliquots and averaging the counts result in a dilution factor of 10, as explained in Example 2b.

2b. Duplicate 2-mL aliquots are plated with SCD agar for the TAMC test. After incubation, 2 CFU are recovered on one plate, and 1 CFU is recovered on the duplicate SCD agar plate. The average recovery is therefore 1.5 CFU, which is rounded to 2 CFU. (*Note*: Never report tenths of a microbial colony. Always round the number of CFU recovered to the nearest whole value.)

The following calculation applies in order to report the result per 1 mL basis:

The average recovery of 2 CFU is multiplied by the dilution factor 10 [10 mL sample ÷ 200 mL total volume (1:20 dilution) × 2 mL aliquot × 10 (dilution factor) = 1]. By increasing the volume of the aliquot plated to 2 mL (1:20 sample dilution), we are able to increase the test sensitivity and achieve a dilution factor of 10. The result reported is then 20 CFU per milliliter of product. If no colonies are detected, the result is reported as < 10 CFU per milliliter.

Dilution factor calculation:

10 mL of sample into 190 mL diluent (1:20 dilution) × 2 mL (aliquot plated) × DF (dilution factor) = 1 (result per milliliter). Therefore, DF = 10.

Example 3: A 10-mL sample aliquot is diluted in 390 mL of buffer to make a 1:40 sample dilution.

3a. Duplicate 1-mL aliquots are plated with SCD agar for the TAMC test. After incubation, 2 CFU are recovered on one of the SCD agar plates and 5 CFU on the duplicate SCD agar plate. Because volumes equal to 1 mL were plated, the dilution factor is 40 [10 mL sample ÷ 400 mL total volume (1:40 dilution) × 1-mL aliquot × 40 (dilution factor) = 1]. The calculated average of 3.5 CFU is rounded to 4 CFU, which is then multiplied by the dilution factor 40. Therefore, the result is reported as 160 CFU per milliliter of product.

Dilution factor calculation:

10 mL of sample into 390 mL diluent (1:40 dilution) × 1 mL (aliquot plated) × DF (dilution factor) = 1 (result per milliliter). Therefore, DF = 40.

3b. Duplicate 4-mL aliquots are plated with SCD agar for the TAMC test, and duplicate 2-mL aliquots are plated with SDA for the TYMC test. After incubation, 2 CFU are recovered on one SCD agar plate, and no colony is recovered on the duplicate SCD agar plate. No colonies are recovered on the duplicate SDA plates.

The following calculation applies in order to report results per milliliter basis:

For TAMC: The average of 1 CFU is multiplied by the dilution factor 10. By plating 4 mL from a 1:40 dilution, the test sensitivity is increased, and a dilution factor of 10 is achieved. The result is reported as 10 CFU per milliliter of product.

Dilution factor calculation:

10 mL of sample into 390 mL diluent (1:40 dilution) × 4 mL (aliquot plated) × DF (dilution factor) = 1 (result per milliliter). Therefore, DF = 10.

For TYMC: Because only 2 mL were plated from the 1:40 dilution, the dilution factor is 20 in order to report results per milliliter basis [10 mL sample ÷ 400 mL total volume (1:40 dilution) × 2 mL aliquot × 20 (dilution factor) = 1]. Because no colonies were recovered, the TYMC result is reported as < 20 CFU per milliliter of product.

Dilution factor calculation:

10 mL of sample into 390 mL diluent (1:40 dilution) × 2 mL (aliquot plated) × DF (dilution factor) = 1 (result per milliliter). Therefore, DF = 20.

Test Controls

When performing a microbial limit test, all media used must be evaluated for sterility and growth-promoting properties, a topic addressed in detail in Chapter 7 of this book. These quality control tests should be performed prior to using the media for product evaluation of microbial contamination. In addition, at the time of testing, settle plates should be exposed in the laminar flow hood for evaluation of suitability of the environment for aseptic work. All test diluents and materials must also be evaluated for sterility as test-negative controls. This is done by plating the test diluent (without the product) with the same lots of media used for testing the product. Be sure to use the same lot of sterile pipettes and spreaders (if applicable) used to test the product. Carry out the same manipulations used to prepare the sample, and incubate the test-negative controls alongside the sample test plates.

TAMC and TYMC Tests via Membrane Filtration Method

The USP membrane filtration method used for microbial limit testing is an adaptation of the USP sterility test described in Chapter <71>. Given the fact that most, if not all, product material will be filtered through the membrane filter, there is minimum product interference, if any. This fact relies on the physical retention of microorganisms on the membrane filters and the assumption that antimicrobial agents that might be present in the sample will pass through the filter into the filtrate.

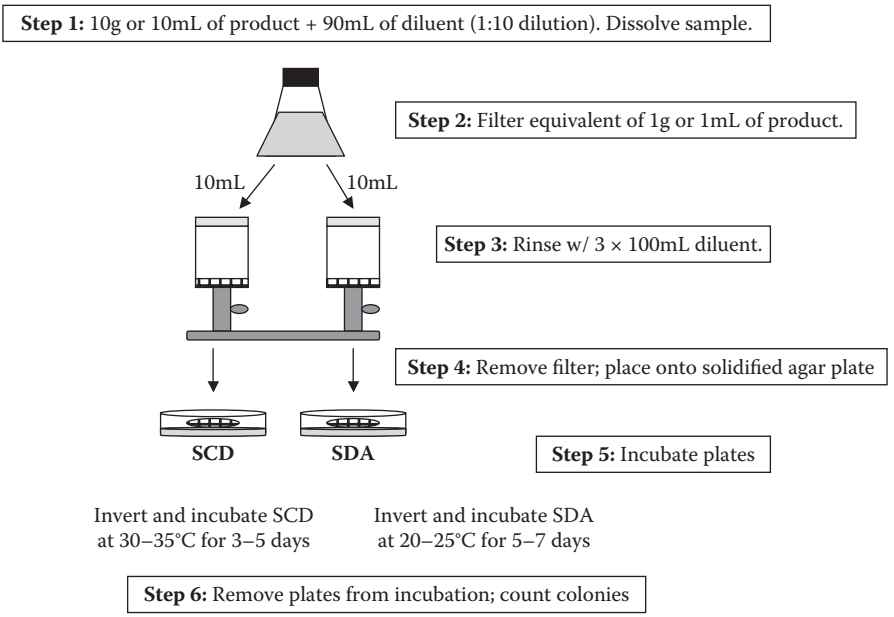


FIGURE 3.2 TAMC and TYMC via membrane filtration method.

In order to perform the TAMC and TYMC tests via membrane filtration, transfer two aliquots of the sample preparation, in buffer or broth diluent, into two sterile filtration units containing 0.45-µm membrane filters. Typically, 10 g or 10 mL of product is added to a diluent to make a 1:10 sample dilution, and 10-mL aliquots, each representing 1 g or 1 mL of the product, are filtered through separate sterile filtration units. Alternatively, the entire sample preparation representing 10 g or 10 mL of product may be filtered for increased test sensitivity.

After filtering the sample preparations, rinse each membrane filter with a minimum of three 100-mL portions of the chosen diluent. Using sterile forceps, aseptically remove the membrane filters and place them onto the surface of solidified agar plates; use SCD agar medium for the TAMC test and SDA medium for the TYMC test. Incubate the SCD agar plate at 30–35°C for 3–5 d and the SDA plate at 20–25°C for 5–7 d. At the end of the incubation period, enumerate isolated colonies with the aid of an instrument such as a colony counter or an illuminator with magnifying lens. If no colonies are present, report results as 0 CFU per gram or milliliter of product. Alternatively, if the equivalent of 10 g or 10 mL of sample was processed, report results as 0 CFU per 10 g or 10 mL of product. See Figure 3.2 for an outline of the test.

Test Controls

When performing a microbial limit test by membrane filtration, all media used must be evaluated for sterility and growth-promoting properties, discussed in Chapter 7 of this book. These quality control tests should be performed prior to using the media for product evaluation of microbial contamination. In addition, at the time of

testing, settle plates should be exposed in the laminar flow hood for evaluation of the suitability of the environment for aseptic work. All test diluents and materials must also be evaluated for sterility as test-negative controls. This is done by filtering the test diluent (without the product) and plating the membrane filters with the same lots of media used for product testing. Be sure to use the same lot of filters/filtration units and sterile disposables used to test the product. Also, perform forceps negative control (dip in TSB and incubate alongside the product samples) to ensure sterility of the materials used to handle the membrane filters. Carry out the same manipulations used to prepare the sample and incubate the test-negative controls alongside the sample test plates.

TAMC Test by the Multiple Tube Method

The multiple tube method is also known as the most probable number (MPN) test. This method can be used to determine the TAMC in a sample. The MPN test is not considered a precise or accurate method, and hence leads to potentially unreliable test results. This method is not recommended for fungal count determination because enumeration of mold is even more unreliable when compared to cell counts for yeast and bacteria. The MPN method should be used only in case no other method is available or suitable for the type of product being tested.

Procedure

Into each of 14 test tubes, place 9–10 mL of TSB. Arrange 12 tubes in four sets of 3 tubes each. Pipet 1 mL of the sample preparation, dissolved or homogenized in the proportion 1:10, into each of the three tubes in the set labeled "100," and into another tube labeled "A" in order to prepare 100 mg or 0.1 mL of sample per tube. From tube "A," pipet 1 mL into each of the three other tubes in the set labeled "10," and into another tube labeled "B" in order to prepare 10 mg or 0.01 mL of sample per tube. From tube "B," pipet 1 mL into each of the three tubes in the set labeled "1," which will contain a final product concentration of 1 mg or 0.001 mL per tube. Keep three TSB tubes from the fourth set as negative controls. Incubate the triplicate sample preparation test tubes from sets "100," "10", and "1" at 30–35°C for not more than (NMT) 3 d. After incubation is complete, observe the broths for turbidity. If reading of results is difficult due to product turbidity, subculture a portion of the suspect sample into fresh TSB and further incubate at 30–35°C for 1–2 d. The three TSB negative control tubes must remain clear. At the end of the final incubation period, the observations in the product-inoculated tubes are evaluated and interpreted as shown in Table 3.1. The final score represents the MPN of microorganisms per 1 g or 1 mL of product. Test confidence limits are also provided and should be taken into consideration when evaluating whether the product meets test specifications for microbial contamination. See Figure 3.3 for an outline of this test.

TABLE 3.1
Most Probable Number of Microorganisms

Number of Tubes in Which Microbial Growth is Observed for Each Quantity of the Test Article				
Set "100" (0.1 g or mL per tube)	Set "10" (0.01 g or mL per tube)	Set "1" (0.001 g or mL per tube)	Most Probable Number of Microorganisms per Gram or Milliliter of Product	95% Confidence Limits
3	3	3	>1100	—
3	3	2	1100	200–4000
3	3	1	460	90–1980
3	3	0	240	40–990
3	2	3	290	90–990
3	2	2	210	30–400
3	2	1	150	30–380
3	2	0	93	18–360
3	1	3	160	30–380
3	1	2	120	30–360
3	1	1	75	17–199
3	1	0	43	9–181
3	0	2	64	16–181
3	0	1	38	9–104
3	0	0	23	5–94
2	3	1	36	9–94
2	3	0	29	9–94
2	2	2	35	9–94
2	2	1	28	9–94
2	2	0	21	5–40
2	1	2	27	9–94
2	1	1	20	5–38
2	1	0	15	4–38
2	0	2	20	5–38
2	0	1	14	4–35
2	0	0	9	2–35
1	3	0	16	5–38
1	2	1	15	5–38
1	2	0	11	4–35
1	1	1	11	4–35
1	1	0	7	1–20
1	0	2	11	4–35
1	0	1	7	1–17
1	0	0	4	<1–17

Continued

TABLE 3.1 (Continued)
Most Probable Number of Microorganisms

Number of Tubes in Which Microbial Growth is Observed for Each Quantity of the Test Article				
Set "100" (0.1 g or mL per tube)	**Set "10" (0.01 g or mL per tube)**	**Set "1" (0.001 g or mL per tube)**	**Most Probable Number of Microorganisms per Gram or Milliliter of Product**	**95% Confidence Limits**
0	3	0	9	4–35
0	2	0	6	1–17
0	1	1	6	1–17
0	1	0	3	<1–10
0	0	1	3	<1–10
0	0	0	<3	0–9

Note: Adapted from harmonized compendial chapters; values rounded to nearest whole numbers.

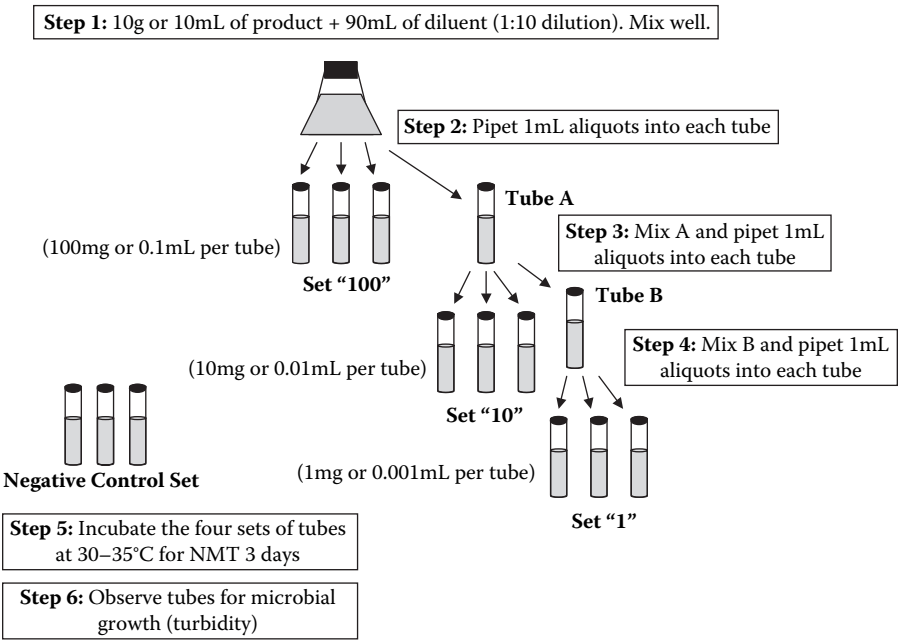


FIGURE 3.3 TAMC via MPN method.

Interpretation of the TAMC and TYMC Test Results

A significant change to the harmonized USP microbial limit tests is in the interpretation of test results. It is well known that microbial contamination is not uniform; hence the greater-than-normal test variability encountered when performing microbial tests for bioburden determination. In order to accommodate for such test variability, the revised USP Chapter <61> allows variability in test results equal to a factor of 2. For example, if the specified microbial limit is 10 CFU, the maximum microbial count that still meets product specifications is 20 CFU. When the specified microbial limit is 100 CFU, the maximum acceptable count is 200, and so on. This change was introduced in an effort to harmonize with the specified EP interpretation of results for microbial contamination.

USP CHAPTER <62>: MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS

The tests described in the USP Chapter <62>, EP section 2.6.13, and JP section 35 are designed to determine whether a nonsterile product complies with established specifications for absence of specified organisms. The new USP Chapter <62> describes the testing of compendial articles for given microbial species that are indicated in individual product monographs or that are listed as possible objectionable organisms in the USP informational Chapter <1111>, Microbiological Quality of Nonsterile Pharmaceutical Products. Included in the chapter are screening tests for *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli*, *Salmonella* spp., *Clostridia*, bile-tolerant Gram-negative bacteria, and *Candida albicans.* This chapter provides tests for the determination of the absence or limited presence of these specified organisms that may be detected under the given test conditions. By clarifying the purpose of this new chapter, the USP implies that some organisms of concern may actually not be detected using the methods listed, and that testing for selected microbial species should not be restricted to those organisms, specified in the chapter. In some cases, testing for absence of specified microbial species, as listed in a USP monograph, may actually have to be supplemented to detect additional objectionable organisms. Additional testing requirements can be developed using the recommendations included in the USP Chapter <1111>. The use of alternate methods, which include automated methods, is encouraged and may substitute the compendial tests as long as suitable method validations are carried out to demonstrate method equivalency. In addition, the presence of any of the designated species may be confirmed by suitable biochemical cultural methods or automated microbial identification systems. Indeed, the FDA has made it clear that testing just to meet compendial requirements and following compendial protocols may not be sufficient to demonstrate a product's microbial quality. On the other hand, it should also be clear that not every type of product or pharmacopeial article need to be tested for absence of all the organisms specified in Chapter <62>. The limits for microbial contamination and absence of specified organisms are either included in individual product monographs or should be established by the manufacturer on the basis of

product type, route of administration, and target patient population. Having clarified the regulatory expectations in terms of testing for objectionable organisms, let us review the contents of the harmonized compendial chapter for detection of specified microbial species.

Besides addition of tests for *Clostridia*, bile-tolerant Gram-negative bacteria, and *C. albicans*, one of the main changes in the testing for specified microbial species is the use of TSB instead of lactose broth for the enrichment tests for *E. coli* and *Salmonella* spp. During the past few years, the use of TSB as an enrichment medium for enterobacter organisms has been discussed in the scientific community and adopted by the EP. Published articles provide evidence that the use of lactose broth as an enrichment medium for *Salmonella* spp. may not be very effective after all. As addressed in an article in the *Pharmacopeial Forum* [3], it is estimated that only 85% of *Salmonella* subspecies IIIb are capable of fermenting lactose. All other *Salmonella* subspecies (I, II, IIIa, IV, V, and VI) do not ferment lactose. The article also emphasizes that both *E. coli* and *Salmonella* spp. grow very well in TSB, a fact that should not be a surprise to microbiologists because glucose, and not lactose, is the preferred sugar for these organisms; even prior to this revision, inoculum preparations for *Salmonella* spp. and *E. coli* for the purpose of method validation have always been performed using TSB. Chapter <62> also includes references to growth promotion and method-suitability testing, topics discussed in Chapter 7 of this book. A summary of the main differences between the old USP microbial limit tests for the presence of specified microorganisms and the new harmonized methods is presented in Appendix B at the end of this chapter.

Sample Preparation for Direct Inoculation Tests

As discussed earlier in this chapter, samples are prepared using methods that have been shown to be suitable for the type of product to be tested, ensuring that the sample preparation itself will not alter any inherent bioburden present in the sample and that any antimicrobial or inhibitory properties are adequately eliminated prior to testing. All steps in the sample preparation must be performed using aseptic techniques and sterile materials.

Chapter <62> refers to Chapter <61> for sample preparation procedures and recommended diluents to be used. Unless otherwise specified in monographs, sample amounts equal to 10 g or 10 mL should be used for testing. However, the USP has realized that, in some cases, there is limited product available for testing. Therefore, it now makes provisions to test smaller amounts of samples, especially when the batch size is very small.

For a direct inoculation method, the product can be added directly into the enrichment broth. Alternatively, the product may be dissolved or dispersed in a buffer solution and then aliquotted into the enrichment broth. If a membrane filtration method is preferred, after the product is filtered through a 0.45-μm membrane filter, the entire filter is placed in the enrichment broth. In most cases, specifications for absence of a specified microorganism is based on a 1-g, 1 mL, or one product unit. The next sections contain specific requirements for each of the tests included in Chapter <62>.

Test for Absence of *Escherichia coli*

A minimum of 1 g or 1 mL of product should be used to test for absence of *E. coli*. Typically, a 10-mL aliquot of a 1:10 sample dilution (equivalent to 1 g or 1 mL of product) is used to inoculate a suitable volume (usually 100 mL) of TSB. Alternatively, the product sample can be added directly into TSB, especially when testing 10 g or 10 mL of product, to make a 1:10 product dilution. If using the membrane filtration method, be sure to filter an amount of product equivalent to a minimum of 1 g or 1 mL of product (or the rinse volume of one product unit), rinse the membrane filter with a buffer solution (3 × 100 mL), and then place the membrane filter in 100 mL of TSB.

After addition of the product (or membrane filter) to TSB, the sample preparation is mixed well, and the TSB sample preparation is incubated at 30–35°C for 18–24 h. Following incubation, aseptically pipet a 1-mL aliquot of the TSB preparation into 100 mL of **MacConkey broth** and incubate at 42–44°C for 24–48 h. Note that the reason for raising the temperature of incubation for isolation of *E. coli* is to provide better selectivity. Following this incubation period, subculture a portion of the inoculated MacConkey broth, using a sterile loop, onto the surface of a **MacConkey agar** plate and incubate the sample preparation at 30–35°C for 18–72 h. Once incubation of the MacConkey plate is complete, observe the agar surface for presence of microbial growth; if any microbial growth is suspected, the presence of *E. coli* must be confirmed or ruled out using suitable microbial identification tests. If no microbial growth is observed or identification tests are negative for *E. coli*, the product complies with the test for absence of *E. coli*. See Figure 3.4 for an outline of this test.

The USP no longer provides colony morphology descriptions for suspected *E. coli* organisms on MacConkey agar or eosin–methylene blue (EMB) agar; the latter is used to confirm the presence or absence of *E. coli* organisms. *E. coli* typically forms brick red, generally mucoid colonies on MacConkey agar, and colonies are sometimes surrounded by a reddish bile-precipitation zone. On EMB agar, *E. coli* gives a characteristic metallic sheen under reflected light and a blue-black appearance under transmitted light. However, biochemical reactions may not be the best method for confirming the presence of *E. coli* organisms, especially if they are in a stressed condition. The metabolic state of the organisms may actually result in a biochemical profile that may lead to misinterpretation of test results. Hence, the statement in the USP that *any growth on MacConkey* agar indicates the *possible presence* of *E. coli* must be confirmed by suitable identification methods.

Test for Absence of *Salmonella* spp.

In the latest revision of USP Chapter <1111>, tests for absence of *Salmonella* spp. in oral dosage forms containing raw materials of natural origin for which antimicrobial treatment is not feasible are based on 10 g or 10 mL of material. Note that testing for *Salmonella* spp. is the only procedure that requires sampling of a minimum of 10 g or 10 mL of product. The product sample can be prepared in a buffer solution or added directly into TSB to ensure that the minimum requirement for product quantity is sampled. If using the membrane filtration method, be sure to filter an amount

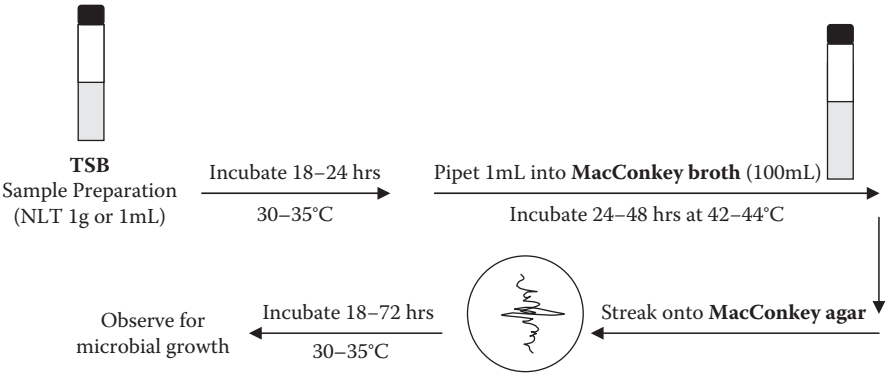


FIGURE 3.4 Test for absence of *Escherichia coli*.

of product equivalent to a minimum of 10 g or 10 mL of product (or the rinse volume of 10 product units), rinse the membrane filter with a buffer solution (3 × 100 mL), and then place the membrane filter in 100 mL of TSB.

After addition of the product (or membrane filter) to TSB, the sample preparation is mixed well and the TSB sample preparation incubated at 30–35°C for 18–24 h. Following incubation, aseptically pipet a **0.1-mL aliquot** of the TSB preparation into 10 mL of **Rappaport Vassiliadis *Salmonella* enrichment broth (RVSEB)** and incubate between 30 and 35°C for 18–24 h. Following this incubation period, subculture a portion of the incubated sample, using a sterile loop, onto the surface of a **xylose, lysine, deoxycholate (XLD) agar** plate and incubate at 30–35°C for 18–48 h.

Once incubation of the XLD plate is complete, observe the agar surface for presence of well-developed red colonies with or without black centers, which could be an indication of presence of *Salmonella* organisms. Suspected growth of *Salmonella* spp. must be confirmed using suitable microbial identification tests. If no microbial growth is observed or identification tests are negative for *Salmonella* spp., the product complies with the test for absence of *Salmonella*. See Figure 3.5 for an outline of this test.

Test for Absence of Bile-Tolerant Gram-Negative Bacteria

Prepare a 1:10 sample dilution using not less than 1 g or 1 mL of product and **TSB** as the test diluent. Homogenize the sample preparation and incubate at 20–25°C for 2–5 h. This preincubation step is designed to resuscitate any bacteria that might be present in the sample without allowing them to multiply. Following the preincubation step, mix the sample preparation well and transfer an aliquot of the sample preparation to a suitable volume of **mossel enterobacteriaceae enrichment broth** (**MEEB**). Typically, a sample aliquot equivalent to a minimum of 1 g or 1 mL of product is added to 100 mL of MEEB. However, the sample amount and volume of broth should be adjusted based on the method suitability studies (method validation).

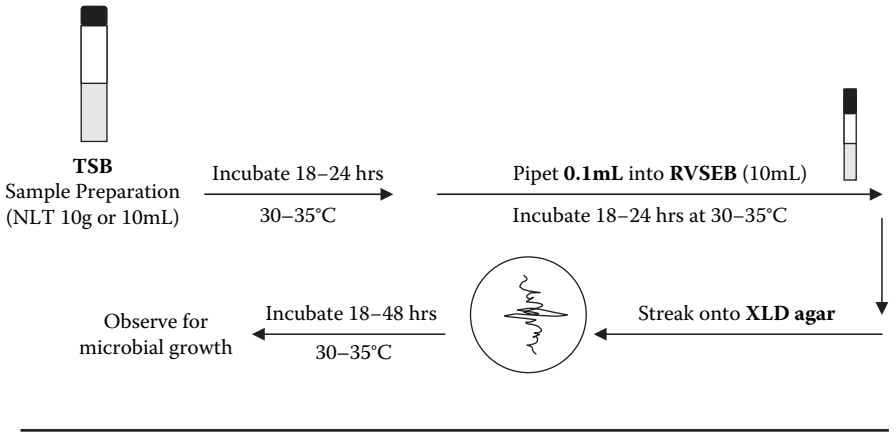


FIGURE 3.5 Test for absence of *Salmonella* spp.

If using the membrane filtration method, be sure to filter an amount of product equivalent to a minimum of 1 g or 1 mL of product (or the rinse volume of one product unit), rinse the membrane filter with a buffer solution (3 × 100 mL), and then place the membrane filter in 100 mL of MEEB.

The MEEB sample preparation incubates at 30–35°C for 24–48 h. Following this incubation period, subculture a portion of the MEEB preparation, using a sterile loop, onto the surface of a **violet red bile glucose (VRBG) agar** plate, and incubate at 30–35°C for 18–24 h.

Once incubation of the VRBG plate is complete, observe the agar surface for presence of red colonies surrounded by a reddish precipitate. If no microbial growth is observed or growth does not meet the previously described colonial morphology, the product complies with the test for absence of bile-tolerant Gram-negative bacteria. See Figure 3.6 for an outline of this test.

Test for Absence of *Pseudomonas aeruginosa*

A minimum of 1 g or 1 mL of product should be used in the test for absence of *Pseudomonas aeruginosa*. Typically, a 10-mL aliquot of a 1:10 sample dilution (equivalent to 1 g or 1 mL of product) is used to inoculate a suitable volume (usually 100 mL) of **TSB**. Alternatively, the product sample can be added directly into the TSB, especially when testing 10 g or 10 mL of product, to make a 1:10 product dilution in TSB. If using the membrane filtration method, be sure to filter an amount of product equivalent to a minimum of 1 g or 1 mL of product (or the rinse volume of one product unit), rinse the membrane filter with a buffer solution (3 × 100 mL), and then place the membrane filter in 100 mL of TSB.

After addition of the product (or membrane filter) to TSB, the sample preparation is mixed well and the TSB sample preparation incubated at 30–35°C for 18–24 h. Following incubation, subculture a portion of the TSB preparation, using a sterile

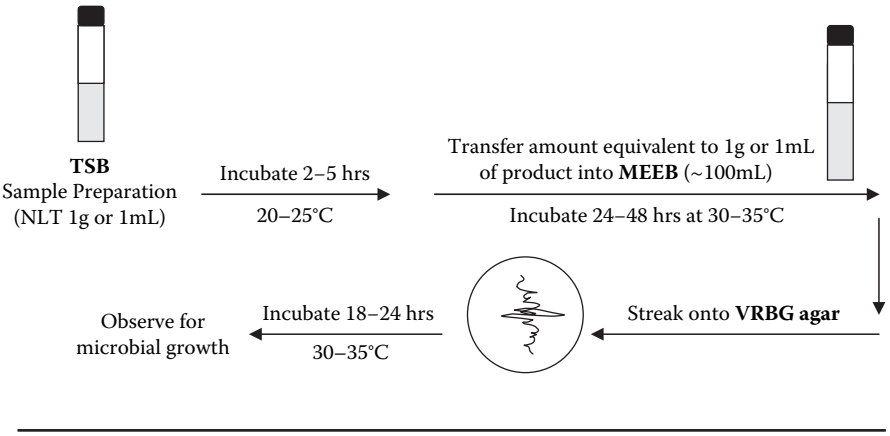


FIGURE 3.6 Test for absence of bile-tolerant Gram-negative bacteria.

loop, onto the surface of a **cetrimide (CET) agar** plate and incubate at 30–35°C for 18–72 h.

Once incubation of the CET plate is complete, observe the agar surface for presence of microbial growth; if any microbial growth is suspected, the presence of *P. aeruginosa* must be confirmed or ruled out using suitable microbial identification tests. If no microbial growth is observed or identification tests are negative for *P. aeruginosa*, the product complies with the test for absence of *P. aeruginosa*. See Figure 3.7 for an outline of this test.

The USP no longer provides colony morphology description for suspected *P. aeruginosa* organisms on CET agar; neither does it recommend confirmation of the presence of *P. aeruginosa* using oxidase tests and detection of fluorescein and pyocyanin on *Pseudomonas* isolation agars. This is because the metabolic state of *Pseudomonas* organisms may actually result in a biochemical profile that may lead to misinterpretation of test results. However, in most cases, colonies of oxidase-positive Gram-negative rods on CET agar, usually having a greenish fluorescence, indicate the presence of *P. aeruginosa*.

Test for Absence of *Staphylococcus aureus*

A minimum of 1 g or 1 mL of product should be used in the test for absence of *Staphylococcus aureus*. Typically, a 10-mL aliquot of a 1:10 sample dilution (equivalent to 1 g or 1 mL of product) is used to inoculate a suitable volume (usually 100 mL) of **TSB.** Alternatively, the product sample can be added directly to the TSB, especially when testing 10 g or 10 mL of product, to make a 1:10 product dilution in TSB. If using the membrane filtration method, be sure to filter an amount of product equivalent to a minimum of 1 g or 1 mL of product (or the rinse volume of one product unit), rinse the membrane filter with a buffer solution (3 × 100 mL), and then place the membrane filter in 100 mL of TSB.

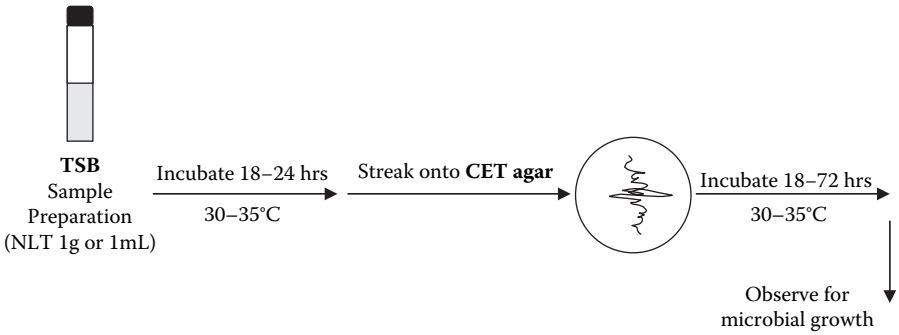


FIGURE 3.7 Test for absence of *Pseudomonas aeruginosa*.

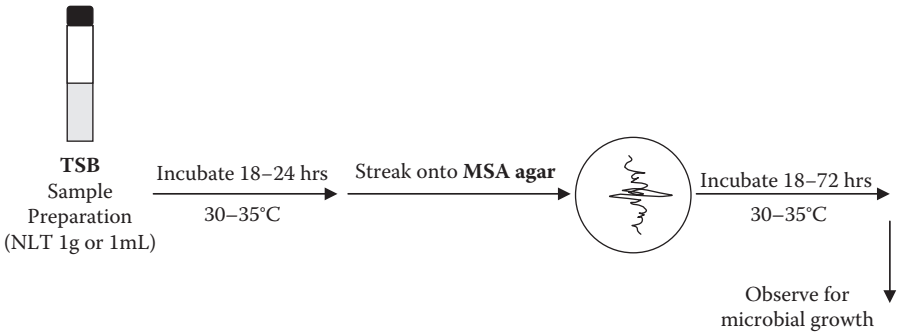
After addition of the product (or membrane filter) to TSB, the sample preparation is mixed well to obtain a homogenous sample and the TSB sample preparation incubated at 30–35°C for 18–24 h. Following incubation, subculture a portion of the TSB preparation, using a sterile loop, onto the surface of a **mannitol salt agar** (**MSA**) plate and incubate at 30–35°C for 18–72 h.

Once incubation of the MSA plate is complete, observe the agar surface for presence of yellow-to-white colonies surrounded by a yellow zone, which could be an indication of presence of *Staphylococcus aureus* organisms. Suspected growth of *S. aureus* must be confirmed using suitable microbial identification tests. If no microbial growth is observed, or identification tests are negative for *S. aureus*, the product complies with the test for absence of *S. aureus*. See Figure 3.8 for an outline of this test.

Test for Absence of *Candida albicans*

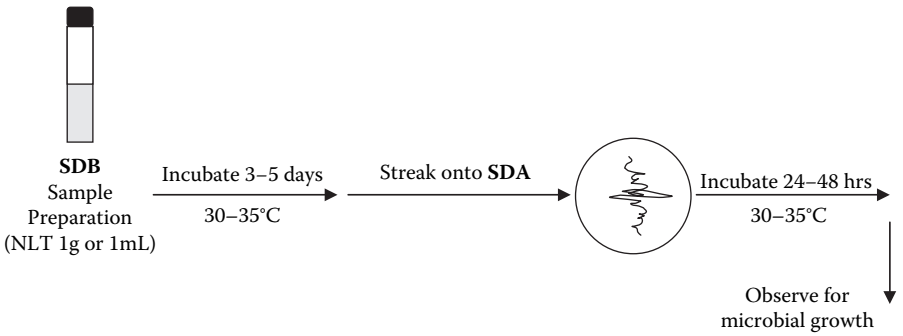
A minimum of 1 g or 1 mL of product should be used in the test for absence of *Candida albicans*. Typically, a 10-mL aliquot of a 1:10 sample dilution (equivalent to 1 g or 1 mL of product) is used to inoculate a suitable volume (usually 100 mL) of **sabouraud dextrose broth** (**SDB**). Alternatively, the product sample can be added directly into the SDB, especially when testing 10 g or 10 mL of product, to make a 1:10 product dilution in SDB. If using the membrane filtration method, be sure to filter an amount of product equivalent to a minimum of 1 g or 1 mL of product (or the rinse volume of one product unit), rinse the membrane filter with a buffer solution (3 × 100 mL), and then place the membrane filter in 100 mL of SDB.

After addition of the product (or membrane filter) to SDB, the sample preparation is mixed well to obtain a homogenous sample and the SDB sample preparation incubated at 30–35°C for 3–5 d. Following incubation, subculture a portion of the SDB preparation, using a sterile loop, onto the surface of an **SDA** plate and incubate at 30–35°C for 24 to 48 h.



- Presence of yellow-to-white colonies surrounded by a yellow zone could indicate presence of *Staphylococcus aureus* which must be confirmed using suitable microbial identification tests.

FIGURE 3.8 Test for absence of *Staphylococcus aureus*.



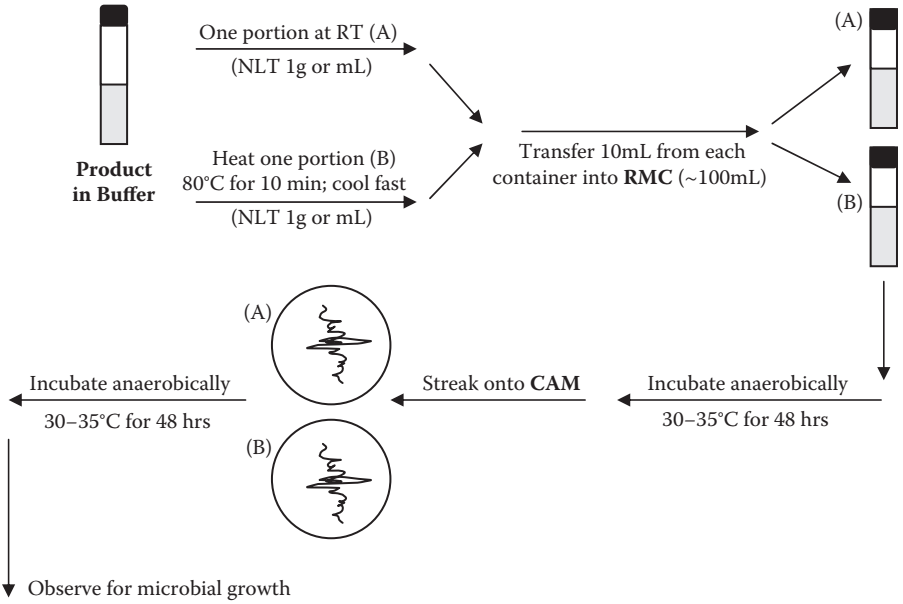
- Presence of white colonies could indicate presence of *Candida albicans* which must be confirmed using suitable microbial identification tests.

FIGURE 3.9 Test for absence of *Candida albicans*.

Once incubation of the SDA plate is complete, observe the agar surface for presence of white colonies, which could be an indication of presence of *C. albicans* organisms. Suspected growth of *C. albicans* must be confirmed using suitable microbial identification tests. If no microbial growth is observed or identification tests are negative for *C. albicans*, the product complies with the test for absence of *C. albicans*. See Figure 3.9 for an outline of this test.

Test for Absence of Clostridia

A minimum of 1 g or 1 mL of product should be used in the test for absence of Clostridia. Typically, two 10-mL aliquots of a 1:10 sample dilution (each equivalent to 1 g or 1 mL of product) prepared in a buffer solution are used for the testing. One por-



- Presence of catalase-negative gram-positive rods (with or without endospores) indicates presence of Clostridia.

FIGURE 3.10 Test for absence of Clostridia.

tion is heated to 80°C for 10 min and then cooled rapidly, whereas the other portion remains at room temperature. Then, the 10-mL aliquots from each buffer sample preparation (heated and not heated) are transferred to separate containers with 100 mL of **reinforced medium for Clostridia** (**RMC**) and incubated under anaerobic conditions at 30–35°C for 48 h.

Following incubation, subculture a portion from each RMC preparation, using separate sterile loops, onto the surface of two separate **Columbia agar medium** (**CAM**) plates and incubate under anaerobic conditions at 30–35°C for 48 h.

Once incubation of the CAM plates is complete, observe the agar surface for presence of microbial growth. The presence of catalase-negative Gram-positive rods (with or without endopores) under anaerobic conditions indicates the presence of Clostridia. If no anaerobic microbial growth is observed or the catalase test is positive, the product complies with the test for absence of Clostridia. See Figure 3.10 for an outline of this test.

Quantitative Test for Bile-Tolerant Gram-Negative Bacteria

Prepare a 1:10 sample dilution using not less than 1 g or 1 mL of product and TSB as the test diluent. Homogenize the sample preparation and incubate at 20–25°C for 2–5 h. The preincubation step is designed to resuscitate any bacteria that might be present in the sample, without allowing them to multiply. Following preincubation,

TABLE 3.2
Most Probable Number of Bile-Tolerant Gram-Negative Bacteria

Test Results			Most Probable Number (MPN) of Bacteria per Gram or Milliliter of Product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	+	+	More than 10^3
+	+	–	Less than 10^3 and more than 10^2
+	–	–	Less than 10^2 and more than 10
–	–	–	Less than 10

Note: Adapted from the harmonized compendial chapter.

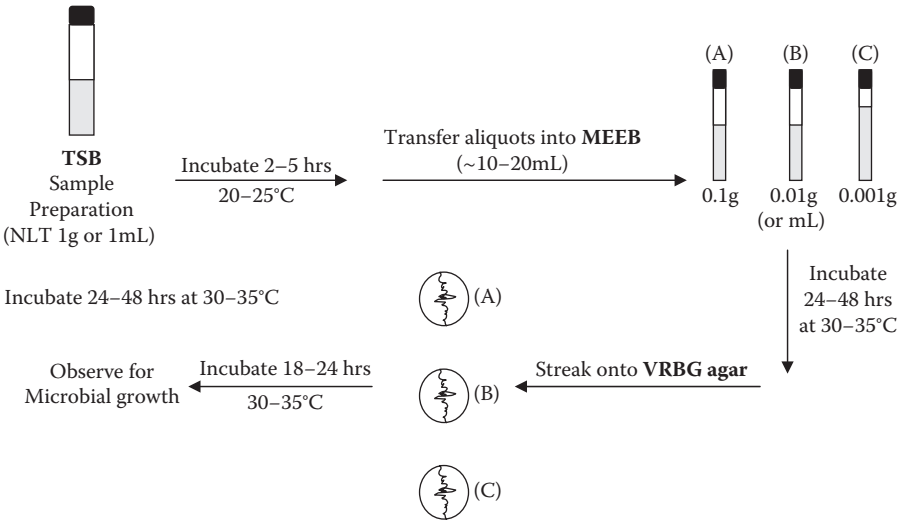
mix the sample preparation well, and transfer aliquots equivalent to 0.1 g, 0.01 g, and 0.001 g (or 0.1 mL, 0.01 mL, and 0.001 mL) into separate containers with a suitable volume of **MEEB**. Typically, test tubes are used for this test, and the volume of medium in each tube is between 10 and 20 mL.

The MEEB sample preparations should be incubated at 30–35°C for 24–48 h. Following this incubation period, subculture a portion of each MEEB sample preparation, using separate sterile loops, onto the surface of **VRBG agar** plates and incubate at 30–35°C for 18–24 h.

Once incubation of the VRBG plates is complete, observe the agar surface for presence of red colonies surrounded by a reddish precipitate (positive). Use Table 3.2 to evaluate the test results obtained and to determine the MPN of bile-tolerant Gram-negative bacteria in the sample. See Figure 3.11 for an outline of this test.

RETESTING

Another change in the USP worth mentioning relates to product retesting guidelines. For the purpose of confirming a doubtful microbial limit test result, a retest using a 25-g specimen of the product is *no longer mentioned* in the USP. Therefore, a company should have a procedure for investigating a test result that fails to meet a given microbial limit specification. Such a procedure may allow for retesting (confirmatory testing), but only up to a certain point, at which the testing ends and the product is rejected. Confirmatory tests (retests) do not replace the original out-of-specification (OOS) or anomalous data unless a laboratory investigation can attribute the suspect result to laboratory error. If the laboratory investigation cannot determine a root cause for the OOS/anomalous result, all values obtained (original and retests) must be reported and taken into consideration when evaluating the microbial quality of the product for batch disposition. According to the regulatory agencies, manufacturing companies are responsible for the quality and safety of their products. Therefore management (quality assurance and well-trained microbiology managers) must use sound scientific principles to decide whether a product should undergo additional testing before reaching a final conclusion on its suitability for release.



- Presence of red colonies surrounded by a reddish precipitate indicates presence of bile-tolerant Gram-negative bacteria.
- Use Table 3.2 to evaluate results and to determine most probable number of bile-tolerant Gram-negative bacteria.

FIGURE 3.11 Quantitative test for bile-tolerant Gram-negative bacteria.

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2. Cundell, A.M. (2002), Review of the Media Selection and Incubation Conditions for the Compendial Sterility and Microbial Limit Tests, *Pharmacopeial Forum*, Vol. 28(6) [November–December].
3. Casey W. M., Heath, J., Goodwin, J. and Hughes, K. (2001), An Alternative Methodology for the General Test Chapter Microbial Limit Test <61>, *Pharmacopeial Forum*, Vol. 27(1) [January–February].

APPENDIX A
Main Changes to USP Chapter <61>

Topic	Obsolete Chapter <61>	Harmonized Chapter <61>
Title and content	Title: "Microbial Limit Tests" Contained tests for microbial enumeration and testing for four objectionable organisms	Title: "Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests" Contains only tests for microbial enumeration; a new chapter <62> was created to address testing for absence of specified microorganisms
Media growth promotion (GP) and method validation	Did not specifically address GP Discussed briefly preparatory testing (method validation) Did not specify inoculum size for preparatory testing Required fresh inocula for preparatory testing Did not provide list of suitable test organisms Did not provide guidance for neutralization studies	Evaluation of GP, and Bacteriostasis and Fungistasis requires that a test sample not differ from test controls by more than a factor of 2 (0.3 log) Reference to method validation replaced by "method suitability verification" considered more appropriate for compendial methods Specifies a low-level inoculum for challenge studies as less than 100 CFUs More flexibility for inocula preparation: allows use of commercial strains, solid or liquid media, potato dextrose agar (PDA) for preparation of *Aspergillus niger*, use of a stable spore suspension, and storage of organism suspension under refrigerated conditions for up to 24 h Provides list of test organisms to be used in GP and method suitability testing Provides list of common neutralizing agents for interfering substances
Sample size/sampling	Allowed for testing of 10 mL or 10 g of material No exceptions mentioned for small batches or limited sample	Allows composite testing of at least 10 articles Sample quantities may be reduced if • Active drug substance is ≤ 1 mg per unit dose (or gram or milliliter of product): sample quantity must not be less than the amount in 10 dosage units or total of 10 g (or mL) of material • Bulk active substance is < 1000 mL (or grams): sample 1% of batch • Batch size is <200 articles: sample two units • Batch size is <100 articles: sample one unit

Sample preparation	Contained sample preparation methods for soluble solids, fluid specimen, water-immiscible fluids, ointments, creams, waxes, and fluid specimens in aerosol form Did not contain a section for transdermal patches	Contains clarifications and alternate sample preparations; includes use of isopropyl myristate (IPM) for fatty products Contains a sample preparation for transdermal patches
Test methods	Provided instructions for pour-plate (PP) and multiple tube method (MPN); referred to USP Chapter <71>, Sterility Tests, for membrane filtration (MF) method Did not acknowledge spread-plate (SP) method Did not specify types of products that are not suitable for these methods Did not have a procedure for testing transdermal patches Did not include guidelines for dealing with products containing antimicrobial properties	Includes method clarifications for PP, MF, and MPN, such as confidence limits for the MPN method, subculturing of suspect tubes from the MPN test for confirmation of results, and the use of single-membrane filters per recovery medium (rather than duplicates) for microbial enumeration tests via membrane filtration method Includes an SP method Clarifies that the tests contained in the chapter are not applicable to products containing viable cells as active ingredients Includes a test method for transdermal patches Contains guidelines on how to deal with products containing antimicrobial properties; defines inhibition as reduction in count by a factor equal to or greater than 2 (0.3 log)
Incubation for TAMC	MF and PP: 48–72 h MPN: 24–48 h	MF, PP, and SP: 3–5 d MPN: NMT 3 d
Enumeration for TYMC	Count only fungal colonies	Count all isolated colonies, including bacteria
Plate count methods	Optimum number of colonies not specified	Specifies NMT 250 CFU for TAMC, and NMT 50 CFU for TYMC
Interpretation of test results	None specified	MPN reported as TAMC; allows a factor of 2 (0.3 log) variability for plate-count methods; includes clarifications with regard to enumeration of microbial colonies
Retest	A 25-g (or mL) retest allowed	No retest

APPENDIX B
Main Changes to Testing for Specified Organism — New USP Chapter <62>

Topic	Obsolete Chapter <61>	New Chapter <62>
Media growth promotion (GP)	Did not specifically address GP	Requires tests to confirm nutritive and selective properties of media
	Discussed briefly preparatory testing (method validation)	Reference to method validation replaced by "method suitability verification"; considered more appropriate for compendial methods
	Did not specify inoculum size for preparatory testing	Specifies a low-level inoculum for challenge studies as less than 100 CFUs
	Required fresh inocula for preparatory testing	More flexibility for inocula preparation: allows use of commercial strains, solid or liquid media, and storage of organism suspension under refrigerated conditions for up to 24 h
	Did not provide list of suitable test organisms	Provides list of test organisms to be used in the challenge studies
Sample size/sampling	Allowed for testing of 10 mL or 10 g of material; no exceptions mentioned for small batches or limited sample	Sample size decreased to minimum of 1 g or 1 mL (except for *Salmonella* testing); references revised/harmonized Chapter <61>; same changes apply
Sample preparation	As described for Chapter <61>	References revised/harmonized USP Chapter <61>; same changes apply
Media preparation	Contained specific instructions for media sterilization	Instructions for sterilization of media removed; companies should rely on validated sterilization cycles
Selective media	Most tests required different types of media to test for absence of selective organisms	Only one medium is now specified for detection of a specified microorganism; more focus on automated methods for microbial identification to confirm presence/absence of a given microbial species
	Subculture performed if enrichment medium turbid (microbial growth visible)	Subculture performed even if enrichment medium clear (no visible sign of microbial growth)
Test for absence of *Staphylococcus aureus*	Incubation period for TSB and selective media: 24–48 h	Incubation period for TSB: 18–24 h; incubation period for selective medium: 18–72 h
	Selective media: Vogel Johnson agar, Baird Parker agar, or mannitol salt agar	Selective medium: mannitol salt agar

Test for absence of *Pseudomonas aeruginosa*	Incubation period for TSB and cetrimide agar: 24–48 h	Incubation period for TSB: 18–24 h; incubation period for cetrimide agar: 18–72 h
Test for absence of *Escherichia coli*	Enrichment medium: Lactose broth (LB) Incubation period for LB and MacConkey agar: 24–48 h	Enrichment medium: TSB Incubation period for TSB: 18–24 h Additional incubation in MacConkey broth at 42–44ºC for 24–48 h followed by subculture onto MacConkey agar incubating at 30–35ºC for 18–72 h
Test for absence of *Salmonella* spp.	Enrichment medium: lactose broth (LB) Incubation period for LB: 24–48 h Use of selenite cystine and tetrathionate broths Streak onto brilliant green agar, XLD agar, and bismuth sulfite agar, and incubate for 24–48 h	Enrichment medium: TSB Incubation period for TSB: 18–24 h Use of Rappaport Vassiliadis *Salmonella* enrichment broth incubating at 30–35ºC for 18–24 h Streak onto XLD agar only and incubate for 18–48 h
Test for absence of Clostridia	Did not include this test	Includes a test for Clostridia using reinforced medium for Clostridia and Columbia agar
Test for absence of bile-tolerant Gram-negative bacteria	Did not include this test	Includes tests for absence and enumeration of bile-tolerant Gram-negative bacteria; tests include a pre-enrichment step for resuscitation of bacteria in TSB; selective media are Mossel enterobacteriacea enrichment broth and violet-red bile glucose agar
Test for absence of *Candida albicans*	Did not include this test	Includes a test for *Candida albicans* using sabouraud dextrose broth and sabouraud dextrose agar

4 Pharmaceutical Waters

TYPES OF WATER FOR PHARMACEUTICAL PURPOSES

Water is the most widely used ingredient in pharmaceutical manufacturing and the main component required for equipment and system cleaning. Therefore, special attention is given to this material in this chapter. Control of its microbial quality is difficult due to its source: municipal and nonmunicipal water systems, which is the major exogenous source of microbial contamination of pharmaceutical waters. It is estimated that there are 70 different types of bacteria in wastewater [1]. Several different types of microbes cross water-treatment barriers and are found in pharmaceutical waters. Most microbial contaminants are Gram-negative bacteria that pose the additional risk of endotoxin contamination of waters used for parenteral production.

Water used as an ingredient for pharmaceutical preparations must meet the requirements for purified water, water for injection, or one of the sterile forms of water covered by a monograph in the pharmacopeia. The USP Chapter <1231>, *Water for Pharmaceutical Purposes* [2], is a useful guide for the pharmaceutical microbiologist because it contains not only information on water testing and minimum water quality standards but also information on the manufacture of the various types of pharmaceutical waters as well as microbial control and validation of water systems. Other useful guides and applicable regulatory documents for pharmaceutical water systems include the FDA's *Guide to Inspections of High Purity Water Systems*, the 21 CFR Parts 210 and 211, and the Baseline Pharmaceutical Engineering Guide, Volume 4: *Water and Steam Guide*. The last-named document, which was prepared by the International Society for Pharmaceutical Engineering (ISPE) with feedback from industry representatives and comments by the FDA, is the current thinking of subject matter experts in relation to engineering of new water and steam systems.

This chapter will focus on regulatory compliance for water systems for the U.S. market, and thus attention will be given to USP and FDA standards. Figure 4.1, adapted from the USP Chapter <1231>, shows the different types of water used for pharmaceutical purposes. In general, pharmaceutical waters can be divided into two groups: bulk water manufactured on-site and packaged water (produced, packaged, and sterilized).

- Purified water: Purified water represents water rendered suitable for pharmaceutical purposes by processes such as distillation, ion exchange, filtration, or reverse osmosis (RO). This type of water is used, for example, as an excipient in the production of nonparenteral preparations, in the cleaning

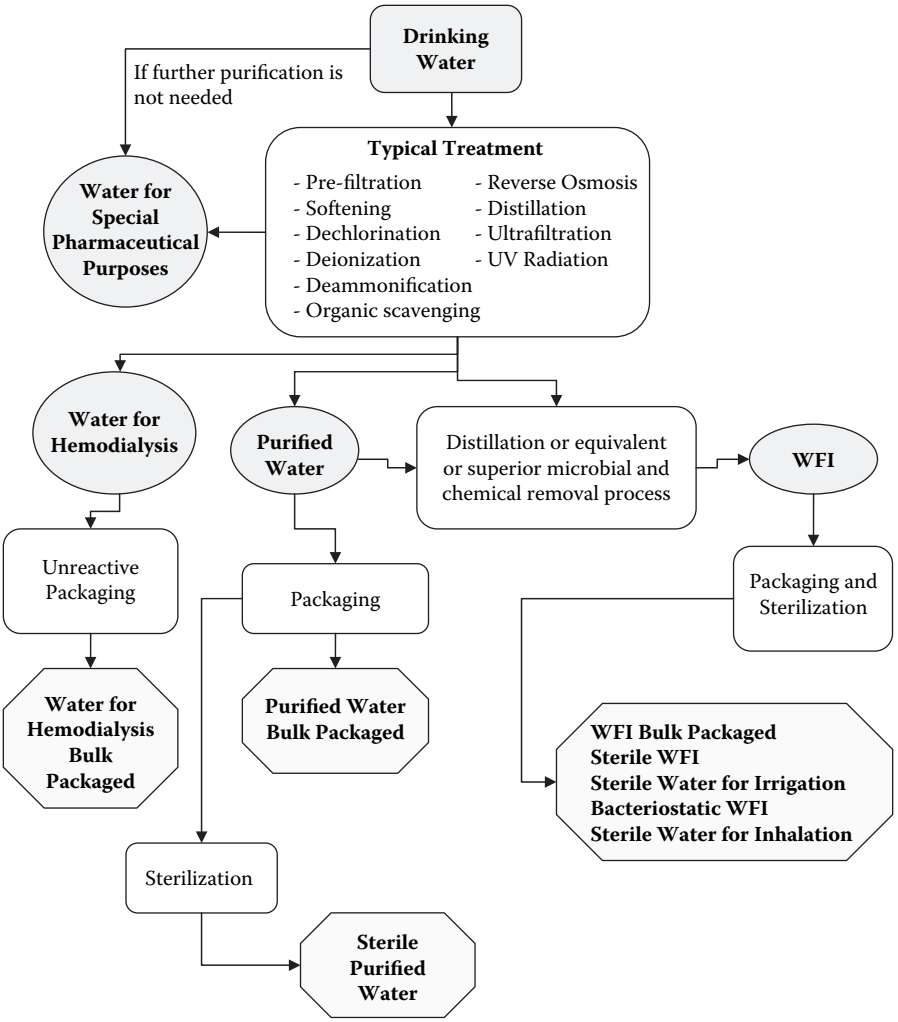

FIGURE 4.1 Types of pharmaceutical waters.

of certain types of equipment (e.g., used in upstream and nonsterile manufacturing processes), and in the preparation of some bulk chemicals. The minimum-quality source (feedwater) for purified water is drinking water as defined by the U.S. Environmental Protection Agency (EPA), the European Union (EU), Japan, or the World Health Organization (WHO).

- Sterile purified water: This type of water is purified water sterilized and suitable for packaging. It does not contain antimicrobial agents, and it is not to be used in preparations intended for parenteral administration.
- Water for injection (WFI): This type of water is prepared using distillation or an equivalent or superior purification process for the removal of microorganisms and chemicals. WFI is used as an excipient in the production of parenteral formulations and for cleaning certain types of pharmaceutical equipment

used in the production of parenteral products (e.g., downstream and aseptic/sterile manufacturing processes). The minimum quality source for WFI is drinking water as defined by the EPA, the EU, Japan, or the WHO.

- Sterile water for injection: This type of water is prepared from WFI that is sterilized and packaged in single-dose containers not more than 1 L in size. It contains no added substances such as antimicrobials. Sterile WFI is intended mainly for use as a diluent for parenteral products.
- Bacteriostatic water for injection: This is sterile WFI containing one or more suitable antimicrobial agents. It is intended mainly for use as a diluent for parenteral preparations. It may be packaged in either single-dose or multidose containers not larger than 30 mL in size.
- Sterile water for irrigation: This type of water is prepared from WFI that is sterilized and packaged. It contains no added substances such as antimicrobials. Sterile water for irrigation meets most, but not all, of the requirements for sterile WFI. Exceptions are the container size (not larger than 1 L in size) and container design (designed to empty rapidly as a single-dose unit); it also does not need to meet the USP <788> *Particulate Matter Test* requirements under small-volume injections.
- Sterile water for inhalation: This type of water is packaged and sterilized WFI for use in inhalators and in the preparation of inhalant solutions. It contains no added substances such as antimicrobials except when used in humidifiers or similar devices.
- Water for hemodialysis: This type of water is produced and used on-site, primarily for the dilution of hemodialysis concentrate solutions. It must not contain added antimicrobials, and is not intended for injection. The minimum quality source for water for hemodialysis is drinking water as defined by the EPA, the EU, Japan, or the WHO and that has been subjected to further treatment to reduce chemical and microbiological components.
- Pure steam: This is generated by heating water above 100°C and vaporizing it in a manner that prevents source water entrainment. Pure steam is intended for use in steam sterilization of equipment and porous loads, and for cleaning the places where condensate directly comes in contact with official articles, product contact containers, and surfaces. Pure steam can also be used for air humidification purposes in controlled manufacturing environments. The minimum-quality source is drinking water as defined by the EPA, the EU, Japan, or the WHO.

The USP Chapter <1231> also addresses nonmonographed analytical waters, such as distilled water and deionized water. Depending on their use, specific testing may be a regulatory expectation. For example, most of the purified water systems used in microbiology laboratories for the preparation of media and reagents are routinely tested for total organic carbon (TOC), conductivity, and bioburden.

The next section of this chapter will focus on the microbiological attributes and bioburden testing of pharmaceutical waters and feedwater. However, readers are encouraged to become familiar with other required microbiological and chemical tests, including *Total Organic Carbon* (USP Chapter <643>), *Water Conductivity*

(USP Chapter <645>), *pH* (USP Chapter <791>), *Sterility Tests* (USP Chapter <71>), and *Bacterial Endotoxins* (USP Chapter <85>). The bacterial endotoxins test has been harmonized (USP, EU, and JP). However, some of the chemical test requirements are different or specific to a given pharmacopeia. Therefore, in order to meet multicompendial chemical quality attributes, tests other than the ones specified in the USP may have to be performed for a given type of water. It is very important for the users (engineers, manufacturers, quality assurance and quality control personnel) to know and understand the global chemical and microbiological requirements for pharmaceutical waters to ensure full compliance with the regulations that apply to their manufacturing processes. Table 4.1 provides an overview of global (US, EU, and JP) compendial microbiological specifications for pharmaceutical-grade waters.

MICROBIAL QUALITY ATTRIBUTES

The quality of bulk waters supplied to manufacturing must meet the quality requirements of the product manufactured. For some applications, meeting the compendial specifications alone may not be sufficient. For example, although a bioburden level is not specified in the various USP water monographs, microbiological testing of water systems is necessary to ensure an acceptable quality of water supplied to manufacturing operations. In general, bulk water monographs in the compendia control the chemical and bacterial endotoxin purity of waters. However, the fact that microbiological testing requirement is not included in the monographs does not diminish the importance of this quality attribute. According to the USP, the reason for not adding a microbial specification for bulk monographed water is because these waters can be used in a variety of applications. In addition, the USP states that a microbial specification would not be appropriate for a clean utility since current testing, using traditional microbiological techniques, takes at least 48 h to complete, and pharmaceutical waters are generally produced by continuous processes. In fact, the failure to meet a compendial microbial specification would call into question the entire water system and the products manufactured with the water.

The FDA understands this dilemma and states in its *Guide to Inspection of High Purity Water Systems* "none of the limits for water are pass/fail limits. All limits are *action limits*. When action limits are exceeded, the firm must investigate the event to find the root cause, take action to correct the problem, assess the impact of the microbial contamination on products manufactured with the water, and document the results of their investigation." In other words, action limits for bioburden of water systems should be treated as *process control levels*. If these levels are exceeded, there is an indication that changes to the system may have taken place, and those should be addressed so that the system can be returned to its state of microbial control. In addition, one must understand that a state of control for one system may be quite different from a state of control for another system. Therefore, the regulatory expectation is for companies to establish their own bioburden action limits/levels and monitor their systems to meet these preestablished levels. Such requirements may include, in addition to a bioburden limit, the absence of certain specified microbial species. As pointed out by the FDA in the *Guide to Inspections of Microbiologi-*

TABLE 4.1
Microbiological Attributes for Compendial Monographed Waters

Water Type	USP31-NF26	EP (2007)	JP XIV
Water (tap and well)			Bioburden (18i): NMT 100 bacteria/mL Coliform (18ii): no coliform bacilli/50 mL
Purified water (bulk)	Bioburden: NMT 100 CFU/mL[a]	Bioburden (2.6.12): NMT 100 CFU/mL[b] Endotoxin (2.6.14): <0.25 IU/mL	
Highly purified water		Bioburden (2.6.12): NMT 10 CFU/100 mL[b] Endotoxin (2.6.14): <0.25 IU/mL	
Sterile purified water	Sterility <71>: complies with test for sterility		Sterility: complies with test for sterility
Purified water in containers		Bioburden (2.6.12): NMT 100 CFU/mL[b]	
WFI (bulk)	Bioburden: NMT 10 CFU/ 100 mL[a] Endotoxin <85>: < 0.25 EU/mL	Bioburden (2.6.12): NMT 10 CFU/100mL[b] Endotoxin (2.6.14): <0.25 IU/mL	Endotoxin: <0.25 EU/mL
Bacteriostatic WFI	Sterility <71>: complies with test for sterility Antimicrobial effectiveness test <51>: complies with AET test Endotoxin <85>: <0.5 EU/mL		
Sterile WFI	Endotoxin <85>: <0.25 EU/mL Sterility <71>: complies with test for sterility	Endotoxin (2.6.14): <0.25 IU/mL Sterility (2.6.1): complies with test for sterility	Endotoxin: <0.25 EU/mL Sterility: complies with test for sterility
Sterile water for inhalation	Endotoxin <85>: <0.5 EU/mL Sterility <71>: complies with test for sterility		
Sterile water for irrigation	Endotoxin <85>: <0.25 EU/mL Sterility <71>: complies with test for sterility		
Water for hemodialysis	Bioburden <61>: NMT 100 CFU/mL Endotoxin <85>: <2 EU/mL	Bioburden (2.6.12): NMT 100 CFU/mL Endotoxin (2.6.14): <0.25 IU/mL	
Pure steam	Endotoxin <85>: <0.25 EU/mL		

[a] Guideline.

[b] Guideline; use membrane filtration, R2A medium, incubation at 30–35°C for 5 d; for WFI, test a minimum of 200 mL.

cal Pharmaceutical Quality Control Laboratories, the specific contaminant, rather than the number, is generally more significant, and this also applies to water for pharmaceutical purposes for certain types of products manufactured.

Indeed, the significance of a bioburden in pharmaceutical waters should be evaluated in terms of the use of the product manufactured, the nature of the product, and the potential harm of the bioburden to the user. For example, a company that manufactures a cream to treat wounds should not allow *Pseudomonas aeruginosa* in their water system. A manufacturing company should also consider whether the bioburden in the water could potentially threaten the chemical quality and stability of the finished product. It is the manufacturer's responsibility to ensure that the water used in the production of pharmaceutical articles not only meets applicable regulations but also is of a microbial grade that will ensure the safety and quality of the final drug manufactured.

Drinking water (potable water) must be free of coliforms and has a requirement for a bioburden level of not more than (NMT) 500 CFU/mL [3]. Other types of water may have to undergo treatment to meet drinking water standards prior to being used as feedwater in a pharmaceutical manufacturing facility. Although the municipal supplier for the feedwater must ensure that the water meets EPA (or other applicable local government agency) quality requirements, routine testing by the pharmaceutical manufacturing company is still needed to confirm that the water received at the plant site meets drinking water requirements for bioburden and absence of coliforms.

For purified water, the compendia recommend an action limit of NMT 100 CFU/mL and for WFI, an action limit of NMT 10 CFU/100 mL. The FDA reminds drug manufacturers that, although these microbiological limits are not specifications, the agency's policy is that any action limit over 100 CFU/mL for a purified water system and any action limit over 10 CFU/100 mL for WFI is unacceptable. In fact, it is the agency's expectation that, if a particular water system is capable of producing water with much better microbial quality than the compendial guidelines, the company should set up bioburden limits that reflect the true capabilities of the system. One must remember that the main purpose of establishing an action limit is to detect adverse trends and implement timely corrective actions to ensure that the water system remains in a state of control.

TESTING OF PHARMACEUTICAL WATERS

Water systems are a significant part of regulatory quality inspections, and companies must routinely monitor the bulk water produced to ensure the chemical and microbial quality of this key pharmaceutical ingredient. Critical parameters that directly affect product quality must be defined and routinely monitored. For water systems producing compendial bulk waters, the chemical and bacterial endotoxin specifications in the compendial monographs constitute the critical parameters. As discussed earlier, although a bioburden specification is not listed in the water monographs, the microbial quality of water systems is viewed as a critical parameter that must be monitored, even though it is difficult to react to the results as microbial quality cannot be monitored in real time. To address this issue, companies establish alert and

action levels as system control parameters and evaluate routine water monitoring data against these levels. Later in this chapter we will discuss this topic along with the various methods for bioburden testing of water systems and how best to react to the data collected.

The USP recommends *Standard Methods for the Examination of Water and Wastewater* [4] as a reference for the sampling and testing of water samples. Most pharmaceutical companies use this reference document to create their standard operating procedures for monitoring of water systems.

Sampling Program

The frequency of testing must be sufficient, and samples should be taken from representative locations in the distribution system in order to demonstrate that the water system is in a state of chemical and microbial control. Typically, the frequency of testing and sampling sites are established on the basis of data generated during validation studies. In the Parenteral Drug Association (PDA) Technical Report No. 13 Appendix C [5], the following testing frequencies for clean utilities are recommended:

- Potable water: Weekly/microbial count and coliform testing
- Purified water: Daily (when in production)/chemistry and microbial testing
- Water for injection: Daily for feedwater to still/microbial, chemistry and endotoxin; daily for return loop/chemistry and endotoxin; weekly rotation for all use points/microbial testing
- Clean steam: Monthly/chemistry and endotoxin testing

Sample Collection and Preservation

Collection bottles for microbial testing must be sterile, and sampling must be performed using aseptic technique. When performing sampling for a type of water that contains residual chlorine or other halogens, a reducing agent must be added to the containers prior to water collection. Sodium thiosulfate at a concentration of 0.1 mL of a 10% solution per 120 mL of sample has been proven satisfactory for neutralizing chlorinated water.

For sample collection, sufficient headspace should be present in the bottle to facilitate mixing prior to testing. This is critical because microbial contamination is not uniformly distributed in a sample. When collecting bulk water at the points of use, the operator must flush the line, allowing a forceful flow of water for about 1–3 min prior to sampling. This will ensure that the sample collected reflects the quality of the water in the system. If a sampling hose is normally used to procure water for manufacturing purposes, the operator *must not remove the hose* when sampling the water for testing. This procedure will ensure that the sample collected is representative of the water used in production and that any biofilm formed in the hose is detected.

If samples cannot be processed within 1 h after collection, they must be stored under refrigerated conditions (2–8°C) until testing is performed. For most accurate data, samples should be processed within 1 h after collection [4]. If this is not possible, they should be kept refrigerated, ideally for a maximum of about 12 h, and not exceeding 48 h [2]. The analysts must be aware that any delay in testing may impact

the test results because microbial viability of potential contaminants may decrease, or in some cases increase, if samples are not stored properly.

Bioburden Testing

The bioburden of waters is evaluated on the basis of the number of CFUs in a fixed sample volume tested. Microbiological testing of drinking water (potable water), which is used as the main source water (feed water) in pharmaceutical manufacturing facilities, is subject to the National Primary Drinking Water Regulations (NPDWR) issued by the EPA. For pharmaceutical-grade waters, there is no official standard recovery method; however, the USP recommends, in the informational Chapter <1231>, the following recovery methods that are derived from the *Standard Methods for the Examination of Water and Wastewater* (4):

- Drinking water
  - Use pour-plate method
  - Test a minimum of 1.0 mL of sample
  - Use plate count agar
  - Incubate at 30–35°C for a minimum of 48–72 h
- Purified water
  - Use pour-plate or membrane filtration method
  - Test a minimum of 1.0 mL of sample
  - Use plate count agar
  - Incubate at 30–35°C for a minimum of 48–72 h
- Water for injection
  - Use membrane filtration method
  - Test a minimum of 100 mL of sample
  - Use plate count agar
  - Incubate at 30–35°C for a minimum of 48–72 h

Over the years, the USP Chapter <1231> has undergone several revisions, and the USP has received many comments from pharmaceutical companies concerning the contents of this chapter. Although the bioburden methods recommended by the USP are not ideal for the detection of stressed and starved organisms, they are still recognized as appropriate techniques for establishing trends in bioburden in water systems in a timely manner. The USP also states that other recovery methods, including media and incubation conditions, and larger sample volumes may be used for the optimal recovery of microorganisms found in various types of water systems. In fact, most highly purified water systems are extremely effective in the removal and prevention of biofilm formation; thus, a sample size of 1.0 mL is not appropriate for testing and trending the microbial quality of the water produced.

When using sample volumes larger than 1.0 mL, the membrane filtration method should be used; a membrane filter with a rating of 0.45 μm is generally the preferred method for testing liquid samples for bioburden. This is especially true for water samples because the filtration process allows retention and recovery of a high number of small cells (e.g., Gram-negative and starved microorganisms) typically found

FIGURE 4.2 The Milliflex® system. (Photo courtesy of Millipore Corporation, www.millipore.com. With permission.)

in water systems. Based on the expected bioburden of the samples collected, most pharmaceutical companies have chosen the membrane filtration method for testing purified waters and the pour-plate method, using a 1.0 mL sample volume, for the testing of feedwater. The Milliflex® system (Millipore Corporation, www.millipore.com) seen in Figure 4.2 is a membrane filtration system that offers faster filtration by using filters that have up to twice the surface area of standard 47-mm membranes. Water samples are processed using sterile filter units that combine a funnel and a grid membrane filter in one device and plated with agar-based media contained in ready-to-use cassettes. Figure 4.3 shows bacterial growth on the membrane filter cassette following a specified incubation period.

Recovery Media

The plate count agar medium recommended by the USP for bioburden testing is also known as standard methods agar, or tryptone glucose yeast agar (TGYA). This is a high-nutrient medium and as discussed earlier, may not be suitable for the recovery of many waterborne organisms that are considered starved. These types of cells are alive but unable to divide and therefore form colonies in/on the agar or on the membrane filter. They are sometimes referred to as "invisible" or "viable but nonculturable." For the recovery of this population of bacteria found in water systems, it is recommended that a low-nutrient medium be used. The choice of medium varies; however, R2A agar seems to be popular and yields good results for isolating and detecting waterborne bacteria. R2A is a low-nutrient medium used for pour-plate, membrane filtration, or spread-plate methods. Some studies performed to compare microbial recovery from water samples using different types of media have indicated that R2A agar often yields higher counts as compared to high-nutrient media such as

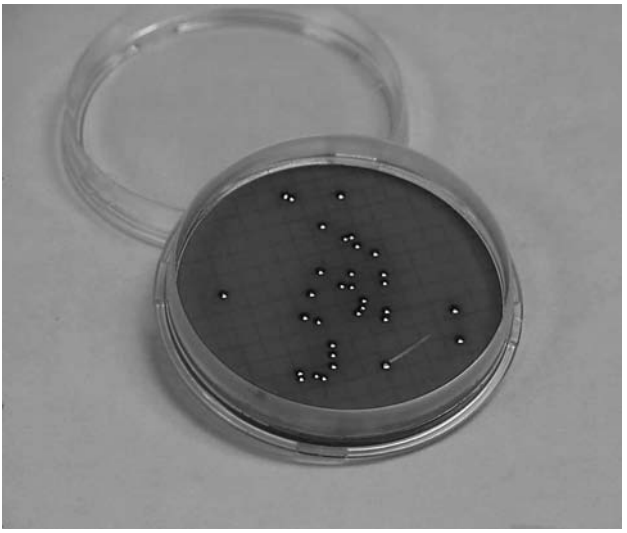

FIGURE 4.3 Milliflex® cassette prefilled with m-Endo LES agar. Coliform colonies appear deep red with a distinct green metallic sheen. (Photo courtesy of Millipore Corporation, www.millipore.com. With permission.)

TGYA, TSA, and m-HPC agar (formerly called m-SPC agar) that are more appropriate for the general isolation and enumeration of heterotrophic and mesophilic bacteria [6]. The recommended bioburden methods that use high-nutrient media specify incubation conditions at 30–35°C for 48–72 h. Typically, when a low-nutrient medium is used, the test plates incubate at 20–25°C for 5–7 d. The longer the incubation and the lower the temperature, the higher the counts obtained because these incubation conditions improve the recovery of waterborne and slow-growing organisms.

The best recovery medium and incubation conditions for testing of water samples has been a hot topic for many years now. Although it is widely known that most methods used cannot recover the invisible microbial flora found in water systems and that bioburden counts recovered are nothing but a rough estimate of the microbial quality of the water produced, scientists often debate the value of having a higher baseline count at the expense of longer test turnaround times. The reality is that detecting higher microbial counts may not add value to early detection of an excursion or an adverse trend. Many of the so-called starved organisms and slow growers become nonviable upon subculturing and therefore cannot be characterized or require resuscitation prior to further characterization. Reviving microbial cells can be accomplished by inoculating the isolated organism in a liquid culture medium and incubating at a moderate temperature. However, this approach adds to the overall testing turnaround time, and may not be practical or considered a value-added activity.

The consensus is that each company should generate data to support the best methodology for testing the microbial quality of its water systems. The decision to use one methodology over another must be based on the company's needs and knowledge of the water system. A company may prefer to use a combination of

methods for a better evaluation of the microbial quality of the water system. For example, a method using a short incubation time may not provide the highest recovery, but it does provide for an early detection of an excursion that can be confirmed with the longer incubation/lower temperature method carried out concurrently. However, most companies seem to opt for methods that yield the highest microbial recovery in the shortest amount of time. Verification of best methodology for a given water system can be performed following experimentation with alternate recovery approaches during or prior to validation of a new water system. In addition to this initial method suitability study, periodic reassessments may be needed for new water systems as the microbial flora gradually stabilizes relative to the original flora detected during system validation. The topic of water system validation is addressed later in this chapter.

Coliform Testing

Coliforms comprise several genera from the Enterobacteriaceae family. Most of these organisms are present in the gut of humans and warm-blooded animals; they are referred to as *indicator organisms* because they indicate the presence of many pathogenic organisms such as parasites, viruses, and protozoa in human or animal feces. In many cases, coliform bacteria are not themselves pathogenic. However, as they are easy to culture and produce gas and acid when fermenting lactose at 35–37°C, these organisms have become the gold standard for fecal contamination testing. Based on their biochemical characteristics, the gas fermentation test for detection of coliform bacilli, which was introduced more than half a century ago, is still used today to test millions of water samples (Figure 4.4). Unlike other coliforms, *Escherichia coli* organisms are usually of fecal origin, and their presence in a water sample is conclusive indication of fecal contamination. Therefore, a presumptive test for fecal coliform bacteria can be confirmed with specific tests for the detection of *E. coli*. These organisms are bile and thermo tolerant, thus having the ability to ferment lactose at 44°C and grow in the presence of bile salts. In addition, they produce characteristic biochemical reactions in certain types of culture media, which can be used for further confirmatory testing.

The *multiple tube fermentation test* (MPN method) using lauryl tryptose broth is generally used to test for total coliforms during the presumptive phase of testing. Brilliant green lactose bile (BGLB) broth is used in the confirmed phase for testing the presence of fecal coliforms [7]. To perform the MPN method, aliquots of the water sample are added to a set of tubes, each containing lauryl tryptose broth and an inverted Durham tube. The tubes are then incubated at 35 ± 0.5°C for 24 h. Following incubation, if gas is noticed inside the inverted tube or acid production is observed (medium turns yellow), or both, the sample is *presumptive* for total fecal coliforms. If no gas or acid is observed following the initial 24-h incubation period, the test samples are incubated for an additional 24 h (for a total of 48 ± 3 h) at 35 ± 0.5°C. At the end of this final incubation period, if no gas or acid is observed, the samples are negative for total coliforms. If acid or gas is or both are present, the sample is presumptive for total fecal coliforms. In order to confirm the presence of fecal coliforms, samples that yielded a positive reaction (gas or acid) in the presumptive

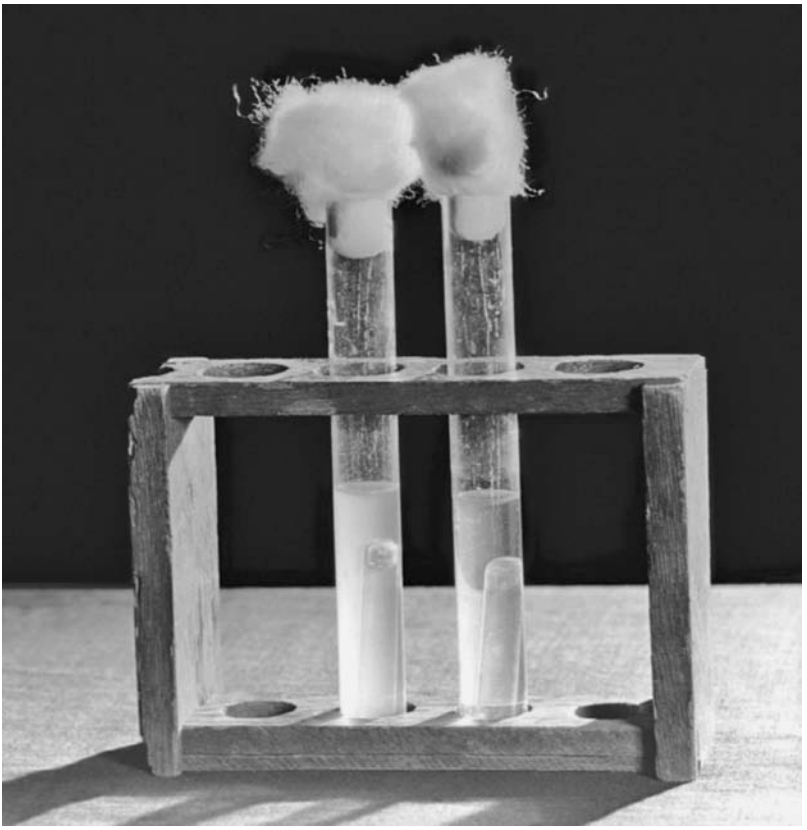

FIGURE 4.4 Historic photograph from 1931, showing equipment used by public health laboratories to analyze water samples: the method involved using broth culture and gas formation to detect *Balantidium coli*. (From Public Health Image Library, Center for Disease Control and Prevention, U.S. Department of Health and Human Services. With permission.)

phase are subcultured into BGLB broth, and another MPN test is performed for a quantitative test. In BGLB broth, fecal coliform bacteria will produce gas; nonfecal coliform bacteria are able to grow but do not produce gas (Figure 4.5). Alternatively, water samples may be processed using lauryl tryptose broth and incubated at 44.5 ± 0.2°C for specific qualitative or quantitative detection of fecal coliforms. Using the MPN tables in *Standard Methods for Examination of Water and Wastewater*, the most probable number of total coliform or total fecal coliform bacteria, or both, can be determined [8].

The *mixed media ONPG-MUG (MMO-MUG) test* is another EPA-approved procedure for total coliforms. To perform this test, a 100-mL water sample is added to a container with MMO–MUG powder, mixed well, and then incubated at 35 ± 0.5°C for 24 h. A positive test is indicated by the development of a yellow coloration in the medium. Other selective media such as M-endo agar LES (Lewis Experimental Station), MacConkey agar, or M-FC medium can be used to screen for fecal coliforms when applying a membrane filtration method [9].

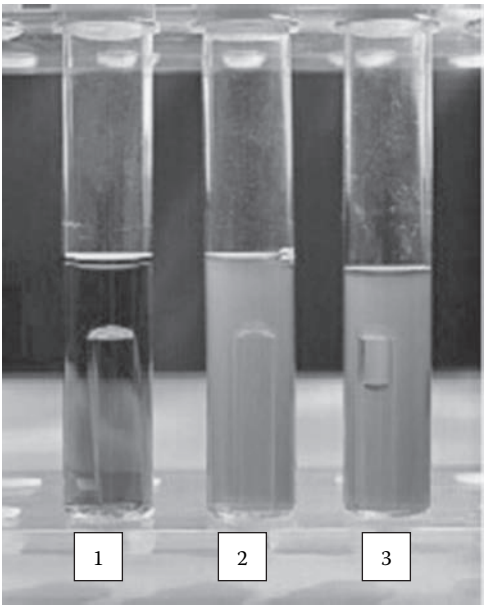

FIGURE 4.5 Brilliant green lactose 2% bile broth (BRILA-broth). Fecal coliforms are capable of growing in the presence of bile and brilliant green fermenting lactose with gas production (Tube 3). Other nonfecal coliform bacteria also grow in this medium, but mostly do not produce gas (Tube 2). Tube 1 is the negative control. (Photo courtesy of Merck KGaA, Darmstadt/Germany. With permission.)

One useful enrichment medium for the screening of indicator organisms in water samples is the presence–absence (P–A) broth [10]. This medium can be used for the screening of total coliform bacteria with test samples incubating at 35 ± 0.5°C for 24–48 h. Following this initial incubation period, observation of a positive reaction (indicated by yellow coloration in the medium and/or gas production) requires confirmation of presence of fecal coliforms as described previously. The P–A test can also be used for screening of other indicator organisms such as *Aeromonas, Pseudomonas*, and fecal *Streptococcus.* This is accomplished by further incubating the test samples (additional 4–5 d at 35 ± 0.5°C) and streaking onto selective agars.

In the JP XIV, a specific test for the testing of coliform bacilli in potable water is presented. This test has a preliminary phase with the water sample incubating in concentrated lactose broth contained in a fermentation tube at 35–37°C for 45–51 h. If no gas production is observed, the test is negative for coliform bacilli. If gas is produced, the incubated culture medium is inoculated into a fermentation tube containing BGLB medium. This confirmatory test sample incubates at 35–37°C for another 45–51 h. If gas is again produced, further identification tests are performed using eosin–methylene blue (EMB) or m-Endo media, lactose broth, and Gram staining. Detection of any Gram-negative asporogenic bacillus indicates the presence of coliform bacilli in the water sample.

The aforementioned methods are traditional ones that have been in use for decades. Several sophisticated and automated instruments for rapid microbial test-

ing are now available in the market, and some are specifically designed for water testing. These automated methods use technologies such as bioluminescence, epifluorescence, impedance, and traditional biochemical reactions. Chapter 9 provides further information on rapid microbial testing instrumentation and how they relate to traditional methods, including its possible impact on historical data and alert/action levels established using traditional culturing methods.

Identification of Waterborne Microorganisms

Information on the types of microbes found in water systems is helpful in identifying the source of contamination. Knowing the typical microbial flora in a water system aids in the evaluation of the effectiveness of system sanitization and in personnel training; it can also serve in early detection of system deterioration.

During system validation, it is a good practice to identify representative isolates in order to establish a baseline microbial flora in the water system. It is also recommended that some of the frequently isolated microorganisms be maintained in the QC laboratory culture collection to be used in studies such as sanitizer efficacy and product bioburden suitability testing.

During routine monitoring of water systems, not every isolated colony needs to be identified. A company should develop a microbial identification program for water isolates that will be both cost effective and makes good business sense. The regulatory agencies do not expect companies to identify every isolate or type of microorganism detected, which is certainly costly and unnecessary. Microbial identification should be performed to provide information for trending purposes and also to assist in manufacturing investigations in case of product contamination.

As a general guideline, when the bioburden detected exceeds the alert level, representative colonies are Gram-stained to evaluate the possible source of contamination. This simple technique provides for microscopic observation of cell morphology (i.e., coccus, rod, single cells, chains, clusters, etc.) as well as for a Gram reaction so that the isolate can be classified as either Gram-positive or Gram-negative.

There are other simple techniques such as the oxidase test and the catalase test as well as checking for spore formation in Gram-positive rods that can be useful for preliminary microbial identification. For most investigations into microbial excursions, these techniques provide sufficient information for data trending purposes. For example, most water isolates are Gram-negative rods, and many are oxidase-positive (e.g., pseudomonads). However, if Gram-positive cocci, which are typically human-borne, are found in a water sample, it may be an indication of poor aseptic technique applied during sample collection or sample testing. In many cases, *Bacillus* organisms are isolated from water samples. Such events, although common occurrences in some companies, should be rare, and when they occur, it could signal poor sampling technique, insufficient flushing of sample ports, or laboratory contamination. In addition, any bacterial contamination found in a hot-water system should be suspect and investigated as potential sample contamination or poor system maintenance.

When the number of recovered organisms exceeds the action level, it is expected that identification to the genus and species level be done and an investigation performed. There is a chance that the action level excursion could be due to sampling

error or inadequate port flushing. However, if there is an indication of true sample-point contamination, the identity of the microbial isolate will be critical for an evaluation of potential product impact.

ESTABLISHING ALERT AND ACTION LEVELS

While establishing alert and action levels for bioburden in pharmaceutical-grade waters, factors such as the intended use, the nature of the product being manufactured, and the effect of the manufacturing process on the fate of viable organisms should be taken into account. For purified water and WFI, chemical and endotoxin (WFI only) specifications are clearly defined in the pharmacopeia. However, as discussed earlier in this chapter, there are no specifications for microbial quality. In lieu of limits/specifications, alert and action levels are established based on system capability and as process-control indicators.

When establishing alert and action levels, one must recognize the difference between *design range* and *operating range*. For example, a purified water system may be designed to deliver water that meets the compendial bioburden guideline of NMT 100 CFU/mL. However, based on company needs and products manufactured, action levels may be set at much lower microbial levels to reflect the allowable operating range that will assure the quality of the final product manufactured. In order to further ensure proper system maintenance and control, a company may yet choose to set alert levels to reflect the normal operating range of the system. Exceeding an alert level should be interpreted as a warning that the system may be drifting away from a state of microbial control. These events do not necessarily require corrective actions. Exceeding an action level should require an immediate investigation into the event so that appropriate corrective actions can be taken to bring the system back into a state of microbial control.

A pattern of multiple and frequent alert level excursions should be treated as an action level excursion and appropriate corrective measures must be taken. Types of immediate actions to take when results exceed action levels often include system sanitization, identification of organisms isolated, evaluation of the possible adverse product impact, and further sampling and monitoring of the water supply as well as other sampling points in the distribution loop. One point to remember is that, although an action level may be exceeded and corrective measures taken, it does not necessarily mean that this raw material is unsuitable for use. This decision will be based on the outcome of the investigation performed to assess the quality of the water produced.

Process controls for water systems may also involve qualitative limits, such as the absence of a particular microbial species. Some companies may choose to monitor specified organisms that are known to cause problems to production equipment through formation of biofilms or compromise the manufactured product. In most cases, companies establish acceptable levels on the basis of the type of microorganisms and the number of colonies detected as the negative impact of a particular microorganism is often greater if present in high numbers.

Besides taking action when alert or action levels are exceeded, a company should establish a system to trend the water-monitoring data for detection of adverse trends. Data that show a deterioration of the microbial quality of the water system over time

require attention in determining the cause and in the implementation of corrective measures. In addition, because alert and action levels should be based on historical data, it is common practice to reevaluate/recalculate these values on an annual basis. In fact, diligent evaluation/interpretation of, and prompt reaction to, data collected are key aspects of an effective management program for water systems.

There is no true consensus on the best approach of setting up alert and action levels based on historical data. The PDA Technical Report No. 13, *Fundamentals of an Environmental Monitoring Program* [5], describes the following approaches:

- Cut-off approach: This method uses the last 100 data monitoring points and uses the 95th and 99th percentile values as the alert and action levels.
- Normal distribution approach: This method calculates the alert level as the mean plus two times the standard deviation (2SD), and the action level as the mean plus three times the standard deviation (3SD) of a population of data points. This method suits a population with high microbial counts best. For low counts, a Poisson distribution should be used.
- Nonparametric tolerance limits approach: Given the fact that clean utilities data (and environmental monitoring data) are not normally distributed and in most cases skewed towards zero counts, non-parametric (distribution-free) statistical methods seem more appropriate for data trend analysis. Nonparametric statistical methods are also simpler and often involve less computational work. Examples of such methods include the *Kruskal-Wallis Analysis of Ranks* and the *Median Test*. To set up alert and action levels, the PDA document recommends using the Tolerance Limits (TL) approach. TL differ from confidence intervals in that they provide an interval within which at least a proportion "X" of a population lies within a certain probability that the stated interval does indeed contain that proportion "X" of the population. For the alert level, TL can be set at a probability value (P) equal to 0.95 and a *Gamma* (γ) coefficient (also a probability value) of 0.95. For the action level, TL can be set using a γ value equal to 0.95 and a P value equal to 0.99.

When choosing the best statistical method for data trending, it is important that the user understands the differences among the tools available and how they apply to the set of data being evaluated. The author recommends the reader to learn more about the approaches discussed in Chapters 4 and 5 of this book and consult with subject matter experts at their companies.

One important point to remember is that the values obtained and the historical data collected are directly related to the type of bioburden methodology chosen for monitoring the water systems. If a company chooses to change the recovery media or even the technology used for detection of microbial contamination, it will have an impact on the test results obtained and how they relate to the established alert and action levels.

VALIDATION OF WATER SYSTEMS

The suitability and performance of water systems to produce water of acceptable chemical and microbiological quality must be validated prior to its use in the produc-

tion of pharmaceutical products. Validation comprises commissioning and qualification activities that should ideally start with system design qualification (DQ). A well-designed water system has a great impact on its longevity, ensuring optimum operation and minimum routine maintenance costs. System design should address the pretreatment and final treatment of the water, the storage and distribution loops, as well as operation, maintenance, and sanitization procedures. Poorly designed systems having areas of stagnant conditions (dead legs), areas of low flow rate, poor-quality feedwater, inadequate sanitization programs, and less-than-adequate material for construction will have an impact on validation efforts and incur long-term maintenance costs to the company. In addition to DQ, a water system validation program qualifies and documents system installation (as IQ), system operation (as OQ), and system performance (as PQ).

The performance qualification (PQ) activities for water systems are unique as they require monitoring of the system over a long period, typically lasting 12 months. Although few problems with the chemical quality of the system are observed over time, variations in the microbial flora as a result of seasonal changes in the feedwater are often noted, thus adding to the challenges of validating a water system. The sampling program for a water system PQ consists of three successive phases:

- *Phase One* begins after the water system is deemed fully operational following operational qualification (OQ) activities. During Phase One, intensive daily sampling of major process points as well as the supply and return points takes place for at least one month. This initial collection of data is valuable and usually sufficient to establish the acceptability of the water system. Data generated can be used to establish system-operating ranges and to create standard operating procedures, to include preventative maintenance and sanitization procedures. At the completion of Phase One, the water may be released for use at risk or with limited applications, or both.
- *Phase Two* of the validation starts at the completion of Phase One, and it may last for another month or two. During this phase of the study, the same testing frequency as well as the number and location of ports sampled during Phase One are maintained. Phase Two is designed to demonstrate consistency in system performance and production of water of a specified quality. At the conclusion of Phase Two, if all test results are acceptable, the water system is considered validated and is released for use in production without restrictions. However, extended performance evaluation, especially to account for seasonal variations in microbial population of the feedwater, is needed. This is accomplished during Phase Three of the validation activities.
- *Phase Three* ensures that additional and frequent monitoring is performed during the first year of the water system's operation. This additional qualification activity is performed to gather sufficient data for trending purposes and for setting meaningful alert and action levels. Typically, the number and frequency of sampling is reduced to reflect use and critical sampling sites. Once the data from the first year of operation are obtained, alert and action levels, previously established based on limited historical/baseline

data or perhaps based on a combination of equipment design capabilities and compendial guidelines, can be reevaluated.

Upon completion of a water system validation, routine monitoring, preventive maintenance, and sanitization must continue in order to control the microbial quality of the water produced.

From a microbiological perspective, the validation of water systems includes testing for bioburden (heterotrophic counts of mesophilic microorganisms), bacterial endotoxins (WFI and pure steam) and, if applicable, screening for organisms of concern. In the past, there was an expectation that hot-water systems should be monitored for thermophiles. However, over the past few years, there has been a shift in paradigm and a realization that the idea that thermophilic bacteria thriving in hot purified water systems is a misconception. There is no doubt that some companies still monitor their hot-water systems for thermophiles, and in the recent past, regulatory inspectors have questioned companies whether or not they monitored their WFI systems for the presence of thermophilic bacteria. The reality is that a hot purified-water system is an extremely hostile environment for these types of microorganisms. Thermophilic and hyperthermophilic microbes require unique environments for their survival and proliferation that include (in most cases) a specific redox potential, extreme pH conditions, temperatures above 70°C, and concentrations of carbon and minerals not found in pharmaceutical waters. For example, most Archaeans are chemolithoautotrophes, and the *Bacillus* spp. (*B. stearothermophilus*, *B. brevis*, and *B. acidocaldarius*), *Clostridium* spp., and *Thermus* spp. that can grow at higher temperatures have special nutritional requirements not found in high-purified water systems. Even if only the spores of *Bacillus* or *Clostridium* organisms were present in the water system, they would not be able to germinate, and would eventually be removed or die off [11]. Therefore, the consensus nowadays among industry experts is that testing for thermophiles in hot pharmaceutical water systems is a non-value-added and costly activity.

The qualification of the bioburden methodologies used for routine water testing (choice of best recovery method) can be incorporated into the water system validation protocol. Typically, at least two criteria (different media and incubation conditions) are evaluated through concurrent testing. Sometimes, the water system is too clean to produce data of statistical significance. In such cases, spike studies using representative waterborne isolates can be performed in the laboratory. Upon data evaluation, if there is a difference of more than 0.5-log (0.3 log harmonized) recovery between the methods evaluated, the medium/incubation conditions chosen for routine testing should be the one that yielded the highest recovery of microorganisms in the shortest amount of time. If the difference is less than 0.5 log (0.3 log harmonized), any of the evaluated methods should be considered suitable for the application.

Remember that the most important aspect of a water-monitoring system is to generate data for early detection of adverse trends. The expectation that a method will be able to detect every type of microorganism present in the water system is unrealistic. A company should strive to develop a bioburden testing program that produces timely results and is cost effective so that management can quickly react to adverse conditions.

MICROBIAL CONTROL AND SANITIZATION

The main challenge for a company in terms of management and maintenance of a water system is prevention of microbial contamination and biofilm formation, a topic that will be discussed in detail in Chapter 10. It is widely accepted in the industry that a continuous and turbulent flow-recirculating hot-water (65–80°C) system is self-sanitizing. Other systems maintained at ambient or cool temperatures can be sanitized using either thermal or chemical means. Other methods such as cold temperatures, distillation, reverse osmosis, filtration, ozonation, and the use of inline ultraviolet (UV) light at a wavelength of 254 nm have also been used to control bioburden in water systems. UV radiation is not considered a sterilization method, and its effectiveness as a sanitizing agent depends on the quality of the water, light intensity, flow rate, contact time, and types of microorganisms present in the water.

Filtration is useful for the removal of not only microorganisms but also particulates and endotoxins (ultrafiltration). Filters used for microfiltration have a porosity range of 0.7–2 μm, and those used for ultrafiltration have a porosity range of 0.005–0.1 μm. Ozone is a strong oxidant, very effective for microbial control and sanitization. For continuous sanitization, ozone is used in storage tanks at levels of 0.02 to 0.1 ppm. In addition, ozone in concentrations as high as 1 ppm is used for periodic sanitization of distribution loops. Chemicals such as chlorine, hydrogen peroxide, and peracetic acid are also used for water sanitization. It is worth noting that any chemical used during sanitization must be removed from the water system prior to releasing water for use in production.

The frequency of sanitization is established during validation exercises; however, additional sanitization may be dictated based on water monitoring and trend results. A good sanitization program must emphasize objections to the storage of wet equipment, the practice of leaving hoses attached to sampling ports without draining, and the placement of sampling hoses on the floor—inadequate practices that can lead to biofilm formation.

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5 Environmental Monitoring

Environmental monitoring (EM) is a requirement as directed in the various regulatory guidance documents in the United States and Europe. Traditionally, microbiological control of manufacturing facilities and personnel was associated with sterile/aseptic processes. However, due to numerous documented cases of contamination of nonsterile formulations, the increase in regulatory requirements and the controlled use of preservative systems have led to an awareness of the need for control and monitoring of nonsterile pharmaceutical production facilities. Environmental monitoring of general quality-control-testing microbiological laboratories has also become standard practice and, in many cases, a regulatory expectation.

An EM program is designed to evaluate the effectiveness of air filtration systems, such as high-efficiency particulate air (HEPA) filters, facility sanitization programs, as well as aseptic training and behavior of manufacturing personnel. As such, an EM program must include monitoring of surfaces, air, and personnel for viable and nonviable particles (air only).

There are several publications that specify the requirements for cleanrooms in terms of design, construction, monitoring, as well as microbial and particulate quality of air and surfaces. The main documents used by pharmaceutical and biopharmaceutical manufacturing companies as references for their EM standard operating procedures are the following:

- International Organization for Standardization (ISO) standard 14644–1, *Cleanrooms and Associated Controlled Environments—Part 1*, *Classification of Air Cleanliness*; and ISO standard 14644–2, *Cleanrooms and Associated Controlled Environments—Part 2*, *Specifications for Testing and Monitoring to Prove Continued Compliance with ISO 14644-1.* European countries have adopted these ISO standards, and in the United States, both documents replaced the Federal Standard 209 in November 2001.
- ISPE Baseline Guide Volume No 6, *Biopharmaceutical Manufacturing Facilities.*
- ISPE Baseline Guide Volume No. 3, *Sterile Manufacturing Facilities.*
- FDA Guide—*Sterile Drug Products Produced by Aseptic Processing* (2004).

- European Commission (EC) Eudralex, Volume 4, *Guidelines to Good Manufacturing Practice*, *Medicinal Products for Human and Veterinary Use*, *Annex 1, Manufacture of Sterile Medicinal Products* (2008).
- USP31–NF26 Chapter 1116, *Microbial Evaluation and Classification of Cleanrooms and Other Controlled Environments.*
- PDA Technical Report No. 13, *Fundamentals of an Environmental Monitoring Program* (September/October 2001).

CLEANROOM CLASSIFICATION

Cleanrooms are classified according to the level of air cleanliness they are able to achieve and maintain. Before ISO 14644–1 was adopted in the United States, cleanrooms were defined in simple terms according to the Federal Standard 209E, *Airborne Particulate Cleanliness Classes in Cleanrooms and Clean Zones*. This standard, first published in the United States in 1963 and last revised in 1992 (revision 209E), classified a cleanroom by the number of nonviable particles of 0.5 μm or larger contained in a cubic foot of air. Cleanrooms were classified into six classes—Class 1; Class 10; Class 100; Class 1,000; Class 10,000; and Class 100,000. A Class 100 cleanroom was designed to not exceed a count of 100 particles of 0.5 μm or larger in a cubic foot of air; a Class 1,000 room was designed to not exceed a count of 1,000 particles of 0.5 μm or larger in a cubic foot of air, and so on.

Within the last 20 years, country-specific cleanroom standards were adopted, which made it very difficult for pharmaceutical companies with worldwide presence or product markets to have a manufacturing operation that could meet the various international requirements. In an attempt to harmonize cleanroom standards and contamination control, ISO and the European Committee for Standardization (CEN) have been working on the development of two families of documents: The ISO 14644 standards on cleanrooms, and the ISO 14698 standards on biocontamination control (see Table 5.1). One of these documents, ISO standard 14644–1, defines cleanrooms for various particle sizes ranging from 0.1 to 5.0 μm, and divides cleanrooms into nine types, from ISO 1 to ISO 9. Comparing the Federal Standard 209E with the ISO classification system, a cleanroom Class 100 is equivalent to ISO 5; a cleanroom Class 10,000 is equivalent to ISO 7; and a cleanroom Class 100,000 is equivalent to ISO 8 classification. Adopted by many countries, including the United States and the European Union, the ISO standard 14644–1 classifies controlled environments in terms of particles per cubic meter without differentiating between viable and nonviable particulate count. It is in the United States and in the European regulatory guidance documents as well as in the USP Chapter <1116> that the acceptable number of viable particles per cubic meter can be found. However, these bioburden values are only guidelines, and the regulatory agencies strongly recommend companies to establish their own microbiological levels based on the nature of their operations.

Although the U.S. and the European cleanroom regulatory guidance documents contain similarities to include discussion on isolator systems, there are still key differences that impact users. For example, the EC *Guide to Good Manufacturing Practice, Annex 1*, defines cleanrooms in terms of grades (namely, grades A to D) and addresses two particle sizes: 0.5 and 5.0 μm. Grade A environments apply to

TABLE 5.1
ISO Cleanroom and Biocontamination Control Standards

Document Number	Document Title	Document Revision Date/Status
ISO 14644-1	Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness	1999/Published
ISO 14644-2	Cleanrooms and associated controlled environments—Part 2: Specifications for testing and monitoring to prove continued compliance with ISO 14644-1	2000/Published
ISO 14644-3	Cleanrooms and associated controlled environments—Part 3: Test methods	2005/Published
ISO 14644-4	Cleanrooms and associated controlled environments—Part 4: Design, construction, and start-up	2001/Published
ISO 14644-5	Cleanrooms and associated controlled environments—Part 5: Operations	2004/Published
ISO 14644-6	Cleanrooms and associated controlled environments—Part 6: Vocabulary	2007/Published
ISO 14644-7	Cleanrooms and associated controlled environments—Part 7: Separative devices (clean air hoods, gloveboxes, isolators, and minienvironments)	2004/Published
ISO 14644-8	Cleanrooms and associated controlled environments—Part 8: Classification of airborne molecular contamination	2006/Published
ISO 14644-9	Cleanrooms and associated controlled environments—Part 9: Classification of surface particle cleanliness	Under development (status date: May, 2008)
ISO 14698-1	Cleanrooms and associated controlled environments—Biocontamination control—Part 1: General principles and methods	2003/Published
ISO 14698-2	Cleanrooms and associated controlled environments—Biocontamination control—Part 2: Evaluation and interpretation of biocontamination data	2003/Published
ISO 14698-3	Cleanrooms and associated controlled environments—Part 3: Measurement of the efficiency of processes of cleaning	2003/Published 2004 (Corrigenda 1)

high-risk operations such as filling zones and aseptic connections; a Grade B environment is used for aseptic preparations and filling, and as background for Grade A; Grades C and D are clean areas for carrying out less critical operations. For example, Grade C environments apply to preparation of solutions to be filtered; a *Grade D* environment is used when handling components after washing.

The FDA Guide on *Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacture Practice*, adopts the ISO designation for cleanrooms but considers only particles of size ≥ 0.5 μm. This FDA guide also distinguishes between only two classified areas—critical operations, and *supporting operations*: Class 100 (ISO 5) is used for critical operations, whereas Class 1,000 (ISO 6), Class 10,000 (ISO 7), or Class 100,000 (ISO 8) can be used for supporting operations. In addition,

there is no equivalent to the EC Grade D in the FDA or USP documents addressing cleanrooms.

In Japan, the other major player in terms of global harmonization of pharmaceutical manufacturing standards besides the United States and Europe, cleanroom technology was introduced from the United States. Therefore, mainly U.S. standards have been applied in Japan. However, through the ongoing effort of global standardization, the Japanese Industrial Standards (JIS) on cleanroom has also adopted ISO standards—JIS B 9919:2004*, Design, Construction and Start-up for Cleanrooms*, which corresponds to ISO 14644-4, and JISB 9920:2002, *Classification of Air Cleanliness for Cleanrooms*, which corresponds to ISO 14644-1. Tables 5.2 through 5.6 summarize and compare the main global cleanroom standards and guidelines.

Occupancy State

The environmental conditions in a cleanroom are defined in terms of the occupancy state as follows:

At-rest or static: Absence of normal operations, no equipment operating, and no personnel present; at-rest conditions should be met after a short "clean up" period (EC recommends 15–20 min) after completion of operations.
Operational or dynamic: Normal operations, for example, equipment operating, personnel present, and/or ongoing process or simulated process.

According to the European standards, requirements to meet a given room classification depend on whether the area is static or dynamic. For example, total particulate count for Grade C static is 352,000/m^3 as compared to 3,520,000/m^3 for Grade C dynamic. In the United States. and according to ISO 14644-1, there is no such distinction. In fact, the FDA's expectation is that environmental monitoring be performed under dynamic conditions for a more meaningful evaluation of the particulate and microbial air, and surface qualities of the production areas that could directly impact product quality and safety.

ROUTINE EM PROGRAM

The purpose of a routine EM program is to detect changes in the environment, sanitization procedures, and/or personnel aseptic behavior that could pose a risk of product contamination. Based on data obtained from routine monitoring, companies can take action to ensure that the environment in the manufacturing facilities remain under particulate and microbial control. Although there are standards for room classification and air cleanliness, there is no standard procedure for the monitoring of air, surfaces, and personnel. Some of the reference documents listed in this chapter, such as the USP Chapter <1116>, provide only recommendations for establishing an EM program for controlled environments. This lack of standardization is intentional because an EM program must be tailored to a given facility and its manufacturing processes. In fact, regulatory inspectors expect companies to use risk analysis and historical data generated at their facility to justify their procedures and acceptance criteria for the monitoring of air, surfaces, and personnel.

TABLE 5.2
Global Cleanroom Standards

	Reference Document					
Standard/Guideline	**ISO (14644-1)**	**FDA (Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing—2004)**	**EC (EU Annex 1 Manufacture of Sterile Medicinal Products, February 2008) Effective Date: March 01, 2009**			**USP31-NF26 Chapter <1116>**
Room Classification	**5**	**5**	**A (at Rest)**	**A (Dynamic)**	**B (at Rest)**	**M 3.5 (Class 100)**
Frequency of EM	Not defined	Should cover all production shifts[a]	Frequent to detect system deterioration	For the duration of critical operations	Frequent to detect system deterioration	Each operating shift
Total particles ≥ 0.5 μm/m^3	3,520	3,520	3,520	3,520	3,520	3,530
Total particles ≥ 5.0 μm/m^3	29	Not stated	20[c]	20[c]	29	Not stated
Microbial active air action level (CFU/m^3)	Not stated	1[b]	Not stated	< 1	Not stated	3
Microbial passive air action level (CFU/4 h)	Not stated	1[b]	Not stated	< 1	Not stated	Not stated
Microbial surface sampling (CFU/contact plate)	Not stated	Not stated	Not stated	< 1	Not stated	3 (including floors)
Personnel monitoring (CFU/glove or gown location)	Not stated	Not stated	Not stated	< 1	Not stated	3 (glove) 5 (gown)

[a] As per the FDA, sample timing, frequency, and location should be carefully selected based on their relationship to the operation performed.

[b] As per the FDA, this area classification should normally yield no microbiological contamination.

[c] Airborne particle classification is ISO 4.8 dictated by the limit for particles ≥ 5.0 μm.

TABLE 5.3
Global Cleanroom Standards

	Reference Document			
Standard/Guideline	**ISO (14644-1)**	**FDA (Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing—2004)**	**EC (EU Annex 1 Manufacture of Sterile Medicinal Products, February 2008) Effective Date: March 01, 2009**	**USP31-NF26 Chapter <1116>**
Room Classification	**6**	**6**	**Not equivalent**	**M 4.5 (Class 1,000)**
Frequency of EM	Not defined	Should cover all production shifts[a]	—	Each operating shift
Total particles ≥ 0.5 μm/m^3	35,200	35,200	—	35,300
Total particles ≥ 5.0 μm/m^3	293	Not stated	—	Not stated
Microbial active air action level (CFU/m^3)	Not stated	7	—	Not stated
Microbial passive air action level (CFU/4 h)	Not stated	3	—	Not stated
Microbial surface sampling (CFU/contact plate)	Not stated	Not stated	—	Not stated
Personnel monitoring (CFU/glove or gown location)	Not stated	Not stated	—	Not stated

[a] As per the FDA, sample timing, frequency, and location should be carefully selected based on their relationship to the operation performed.

TABLE 5.4
Global Cleanroom Standards

Standard/Guideline	Reference document: ISO (14644-1)	FDA (Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing—2004)	EC (EU Annex 1 Manufacture of Sterile Medicinal Products, February 2008) Effective Date: March 01, 2009	USP31-NF26 Chapter <1116>
Room Classification	**7**	**7**	**B (dynamic)/C (at-rest))**	**M 5.5 (10,000)**
Frequency of EM	Not defined	Should cover all production shifts[a]	Frequent to detect system deterioration; dynamic monitoring recommended for critical operations	Each operating shift
Total particles ≥ 0.5 μm/m^3	352,000	352,000	352,000	353,000
Total particles ≥ 5.0 μm/m^3	2,930	Not stated	2,900	Not stated
Microbial active air action level (CFU/m^3)	Not stated	10	10[b]	20
Microbial passive air action level (CFU/4 h)	Not stated	5	5[b]	Not stated
Microbial surface sampling (CFU/contact plate)	Not stated	Not stated	5[b] (no distinction for types of surfaces)	5 10 (floor)
Personnel monitoring (CFU/glove or gown location)	Not stated	Not stated	5[b] (glove print/5 fingers)	10 (glove) 20 (gown)

[a] As per the FDA, sample timing, frequency, and location should be carefully selected based on their relationship to the operation performed.

[b] Dynamic conditions only.

TABLE 5.5
Global Cleanroom Standards

	Reference Document			
Standard/Guideline	**ISO (14644-1)**	**FDA (Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing—2004)**	**EC (EU Annex 1 Manufacture of Sterile Medicinal Products, February 2008) Effective Date: March 01, 2009**	**USP31-NF26 Chapter <1116>**
Room Classification	**8**	**8**	**C (dynamic)/D (at-rest)**	**M 6.5 (100,000)**
Frequency of EM	Not defined	Should cover all production shifts[a]	Monitoring in operation should be performed in accordance with the principles of quality risk management	Twice a week or once a week (nonproduct contact)
Total particles ≥ 0.5 μm/m^3	3,520,000	3,520,000	3,520,000	3,530,000
Total particles ≥ 5.0 μm/m^3	29,300	Not stated	29,000	Not stated
Microbial active air action level (CFU/m^3)	Not stated	100	100[b]	100
Microbial passive air action level (CFU/4 h)	Not stated	50	50[b]	Not stated
Microbial surface sampling (CFU/contact plate)	Not stated	Not stated	25[b] (floors not stated)	Not stated
Personnel monitoring (CFU/glove or gown location)	Not stated	Not stated	Not stated	Not stated

[a] As per the FDA, sample timing, frequency, and location should be carefully selected based on their relationship to the operation performed.

[b] Dynamic conditions only.

TABLE 5.6
Global Cleanroom Standards

	Reference Document			
Standard/Guideline	**ISO (14644-1)**	**FDA (Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing—2004)**	**EC (EU Annex 1 Manufacture of Sterile Medicinal Products, February 2008) Effective Date: March 01, 2009**	**USP31-NF26 Chapter <1116>**
Room Classification	**No Equivalent**	**No Equivalent**	**D (Dynamic)**	**No Equivalent**
Frequency of EM	—	—	Monitoring should be performed in accordance with the principles of quality risk management	—
Total particles ≥ 0.5 μm/m^3	—	—	Not defined	—
Total particles ≥ 5.0 μm/m^3	—	—	Not defined	—
Microbial active air action level (CFU/m^3)	—	—	200	—
Microbial passive air action level (CFU/4 h)	—	—	100	—
Microbial surface sampling (CFU/contact plate)	—	—	50 (floors not stated)	
Personnel monitoring (CFU/glove or gown location)	—	—	Not stated	—

A comprehensive EM program should address the following four main topics:

- Identification of sampling sites and testing frequency
- Instructions for setting alert/action levels
- Description of methods for viable and nonviable particulate monitoring
- Instructions for data analysis and results interpretation

These four topics are addressed in detail in the sections that follow.

Testing Frequency and Sampling Sites

The frequency of routine EM testing must have a direct relationship to the manufacturing operations performed and should be sufficient to allow for meaningful statistical calculations—too much data generated (sites and frequency) can lead to inefficiencies in the quality control testing laboratory in terms of sample processing and data review. Conversely, infrequent testing and inappropriate choice of sites will generate data that will not be meaningful for evaluation of microbial and particulate control in production areas and may not be suitable for trending. In general, sampling sites are chosen based on initial facility and air-handling unit qualification studies (e.g., sites more prone to excursions), room design, proximity to open process operations with potential for product contamination as well as equipment and personnel flow. In terms of testing frequency, a company should increase the frequency of testing as the area of classification increases (e.g., higher cleanroom standards/lower level of particulates). For some types of operations, such as aseptic filling lines, the regulatory expectation is for continuous monitoring during production.

Setting Alert and Action Levels

Maximum levels (action levels) for nonviable particles are defined in the various regulatory and compendial documents for each area classification. Microbial action levels are listed as recommendations in the various industry cleanroom standards. Action levels are those that, when exceeded, indicate that a process has drifted from its normal operating conditions. Therefore, the appropriate response to an action level excursion would be a documented investigation and corrective measure to ensure that the area returns to a state of environmental control. In order to be proactive and better manage the environmental quality of production environments, companies are expected to establish alert levels; exceeding an alert level is an indication that a process may be drifting from its normal operating conditions. Alert levels should be viewed as warning signs and, therefore, these excursions may not require corrective measures. The same methods used for setting up alert and action levels for water monitoring, a topic discussed in Chapter 4, apply to environmental monitoring data; the regulatory expectation is for companies to use historical data when setting up alert and, if appropriate, action levels for their EM programs. These values should be reviewed on an annual basis, and, if needed, adjusted accordingly.

According to the EU guidelines, monitoring for ≥0.5 μm particles is an important diagnostic tool for early detection of problems, such as failure of an HVAC system capable of removing at least 99.97% of airborne particulates 0.3 micrometer or greater in diameter. However, sporadic 0.5 μm particle excursions should be

expected owing to equipment noise and interference with the monitoring process. In such cases, the user is advised to always take immediate additional samples when nonviable particle excursions occur to determine possible false counts and for a better assessment of the nonviable particulate quality of the manufacturing environment.

Test Methods and Equipment

There are several types of methods and pieces of equipment used for the purpose of testing the microbial and nonviable particle quality of controlled environments. For recovery of microorganisms, a general microbiological growth medium such as SCD agar is used so that the total aerobic microbial count (TAMC) can be determined. For specific recovery of fungi, a general mycological medium such as SDA may be used. TAMC plates are typically incubated at 30–35°C for 2–3 d, and plates used for fungal recovery typically incubate at 20–25°C for 5–7 d. When sampling surfaces that have been exposed to disinfectants and sanitizers, the recovery media must be supplemented with a neutralizing agent; when sampling surfaces and air in facilities that have been exposed to antibiotics, the recovery media must be supplemented with an appropriate antibiotic inactivating agent. These neutralizing chemicals help ensure that any inhibitory effect from antimicrobials present in the collected samples is eliminated or minimized for optimum recovery of microbial contaminants.

Over the years, pharmaceutical microbiologists have debated over the need to use SDA for environmental monitoring purposes. In the 2005 draft revision of the USP Chapter <1116>, published in the *Pharmacopeial Forum* (PF) Vo.31(2), the USP acknowledged that SCD agar supports growth of a wide range of bacteria, yeasts, and molds, and therefore, this medium alone should be suitable for monitoring controlled environments. Studies performed by the author have indeed demonstrated that most environmental fungi can be adequately recovered using SCD medium (same as TSA), incubating at 30–35°C for a minimum of 3 d. This testing strategy can be a benefit to an EM program as it minimizes testing costs and decreases test turnaround times. However, because SDA has been used traditionally in environmental monitoring for recovery of fungal isolates, regulatory agencies expect that site-specific studies be performed to demonstrate the suitability of SCD as a medium for the recovery of both airborne bacteria and fungi. This is because, in some cases, specific fungal medium may be required for adequate isolation of site-specific fungi that might be of concern to a particular manufacturing process.

In order to generate data in support of a chosen EM test methodology, airborne microbial recovery studies using various types of media and incubation conditions are carried out simultaneously over a defined period of time. The test method that yields the highest microbial recovery with the shortest incubation period is often chosen for routine EM testing. This type of study can be performed during facility/air handling unit qualification studies, a topic discussed later in this chapter.

Testing devices for recovery of airborne particulates include *active air samplers* and *passive air samplers*. Active air samplers (viable and nonviable particle monitoring) draw a predefined volume of air during the sampling activity. This type of monitoring is considered quantitative because the number of particles recovered can be correlated to the volume of air sampled. Passive air samplers, also referred to as

settling plates, are basically agar-filled Petri dishes kept open and exposed to the environment for the duration of the testing (not to exceed 4 h to prevent media desiccation). Following exposure and incubation at appropriate conditions, recovered colonies are reported as number of CFU per time of exposure. Settling plates are widely used because they are simple and inexpensive air-monitoring devices for qualitative measurements of airborne microbial contamination caused by particle deposition onto surfaces over time due to gravitation. Settling plates are considered by many a better representation of actual contamination events during production as organisms are not forced into the air-sampling device but fall onto the plate by chance due to airflow patterns and personnel movements. In Europe, the use of settling plates is specified as a requirement for environmental monitoring programs. In the United States, the use of settling plates, in addition to active air samplers, is optional.

Surface Sampling for Viable Particles

Most surface sampling and personnel monitoring activities are performed using contact plates (surface area of approximately 25 cm^2) filled with an appropriate recovery medium such as SCD or SDA (supplemented with Tween 80 and lecithin), or Dey-Engley (D/E) medium, the latter considered a universal neutralizing medium for antimicrobials [1]. Contact plates are incubated at the appropriate conditions specified in the test method, and results are reported in terms of number of microbial colonies recovered per 25 cm^2 of area sampled. Figure 5.1 shows an SDA contact plate with fungal growth.

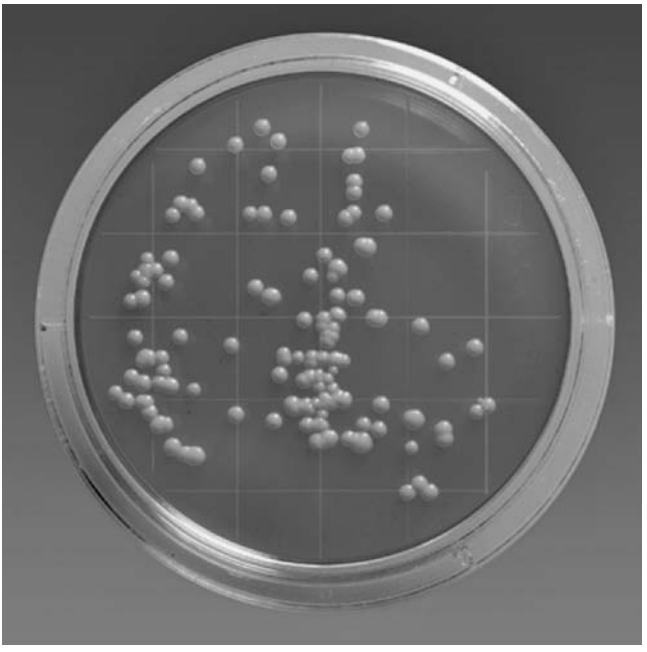

FIGURE 5.1 Contact plate (sabouraud dextrose agar; SDA) with *Candida albicans*. (Photo courtesy of Thermo Fisher Scientific, Remel Products, www.remel.com. With permission.)

FIGURE 5.2 Bacti-Swab® collection and transport systems. (Photo courtesy of Thermo Fisher Scientific, Remel Products, www.remel.com. With permission.)

Contact plates are suitable for sampling flat and regular surfaces. When performing EM of irregular surfaces (e.g., pieces of equipment, tubing, etc.), the best approach is to use a sterile swabs. Certain types of swabs, such as the Bacti-Swab® (Remel Inc., www.remel.com) collection seen in Figure 5.2, and the nylon-flocked QUANTISWAB® (bioMérieux, Inc., www.biomerieux-usa.com) seen in Figure 5.3, are used for these applications. Studies performed have indicated that QUANTISWAB

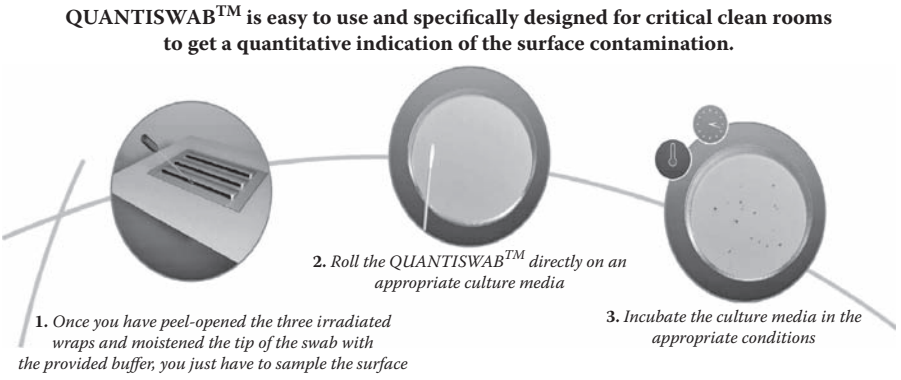

FIGURE 5.3 The Nylon-flocked QUANTISWAB™. (Photo courtesy of bioMérieux, www.biomerieux-usa.com. With permission.)

achieves improved release and recovery of microbes during surface environmental sampling [2]. Swabs should be premoistened with a sterile diluent (e.g., sterile saline solution) for sampling of dry surfaces. The sampling operator should stroke the swab over the desired area using close parallel sweeps while rotating the swab. For best results, the same area should be swabbed again using the same swab, but stroking the swap perpendicular to the initial sweep. After swabbing is completed, the swab may be streaked onto an agar medium or broken into some enrichment medium for a presence/absence test, or broken into a neutralizing diluent, vortexed for about 30 s, and the liquid sample preparation tested by the pour-plate or membrane filtration method for a quantitative measurement. The incubation conditions for the recovery media vary depending on company protocols. However, in general, swab preparations are plated with SCD medium and plates incubated at 30–35°C for 3–5 d. Results are often reported as number of CFU per swab or area sampled (if this information is available).

Sampling of surfaces in a pharmaceutical manufacturing environment often involves monitoring of critical areas that may come in contact with the product. Therefore, surface and personnel monitoring in critical environments should be carried out at the conclusion of operations to minimize disruptions to the process, which could lead to product contamination. After sampling a site with an agar-containing device, it is also crucial to wipe the surface clean with sterile 70% alcohol to remove any residual medium that would promote microbial growth.

Active Air Sampling for Viable Particles

For active air sampling, there are many types of devices available in the market, each having distinct technologies and collection efficiencies. When choosing an air sampler, one must ensure that the equipment is suitable for the area being monitored and that parts can be properly sanitized/sterilized to prevent adventitious contamination during sampling. In addition, the device itself should not interfere with manufacturing operations by causing turbulence in the air around critical areas where product is exposed to the environment, which could lead to increased chance of product contamination.

The types of active air samplers most used in pharmaceutical and biopharmaceutical industries are *impaction* and *centrifugal* devices.

Impaction sampler/slit-to-agar (STA): This type of unit is powered by an attached vacuum source that controls the volume of air sampled. The Mattson–Garvin air sampler (Barramundi Corporation, www.mattson-garvin.com) is an example of such a unit. Air intake is obtained through a slit on the cover dome of the instrument and deposited onto the surface of a slowly revolving Petri dish (150 × 15 mm) containing the nutrient medium. Particles in the air that have sufficient mass will impact on the agar surface. After sampling, the cover is placed back on the Petri dish, and the plate is incubated for a specified amount of time at a specified temperature range. Viable microorganisms grow to form colonies. Results are reported as number of CFU per volume of air (m^3 or ft^3) sampled.

FIGURE 5.4 The SAS 180 air sampler. (Photo courtesy of Bioscience International, www.biosci-intl.com. With permission.)

Sieve impactor: This type of device is designed to hold a standard-size Petri dish containing nutrient agar. It also contains a cover with perforations of predetermined sizes. The air is drawn inside the cover and deposited onto the surface of the agar plate by means of a vacuum pump. After sampling, the cover is placed back on the Petri dish and the plate is incubated at a specified temperature range for a specified time. Results are reported as number of CFU per volume of air (m^3 or ft^3) sampled. The SAS-180 (BioScience International, www.biosci-intl.com) seen in Figure 5.4, the M Air T® (Millipore Corporation, www.millipore.com) seen in Figure 5.5, and the MAS 100 (EMD Chemicals Inc., www.emdchemicals.com) seen in Figure 5.6 are examples of this type of technology for environmental monitoring for airborne microbes. The MAS-100 uses standard 100 mm Petri dishes and collects air samples at 100 L/min (sampling volumes are configurable between 0 and 2000 L). The top section of the unit rotates on its handle to allow for testing at different angles. The MAS-100 is controlled using single-touch "yes" or "no" responses, and it features a 60-min programmable start delay. Therefore, there is no need for a remote control, and personnel can exit the sampling area when the sampling starts. The MAS-100 also features a data port that allows for data transfer to a computer via a special communications cable. Some sieve impactor devices are available with a cascaded series of containers having decreasing size of perforations. These types of air samplers allow for the determination and correlation of particle size and microbial contamination.

FIGURE 5.5 The M-Air T®, a sieve-impaction air sampler with ready-to-use agar cassettes. (Photo courtesy of Millipore Corporation, www.millipore.com. With permission.)

FIGURE 5.6 The MAS-100 air sampler. (Photo courtesy of EMD Chemicals, www.emd-chemicals.com. With permission.)

FIGURE 5.7 The RCS High Flow. (Photo courtesy of Biotest AG, www.biotest.de/. With permission.)

Centrifugal sampler: This type of unit contains a propeller or turbine that pulls a known volume of air into the instrument and then pushes it outward onto a flexible plastic strip containing the nutrient agar. The RCS™ High Flow (Biotest AG, www.biotest.de/), seen in Figure 5.7, uses a special form of impaction principle to collect viable particles at a flow rate of 100 L/min. Rotation of the rotor blades in the head of the instrument, where the medium strip is inserted (see Figure 5.8), generates an air flow that enables airborne particles to be impacted by centrifugal force onto the agar medium. After sampling, the agar strip is placed back into its original sterile plastic cover and incubated for a specified time at a specified temperature range. Colonies formed are counted, and results are reported as number of CFU per volume of air (m^3 or ft^3) sampled. Studies performed indicate that both the RCS Plus and RCS High Flow show high collection efficiency, primarily with smaller particles (0.5 μm), and that collection efficiency of larger particles is not significantly higher than the generally accepted standard [3]. Features of the RCS High Flow include air direction rings to diminish turbulence in unidirectional flow environments, user-selectable sampling volumes, delayed sampling time, and full compliance with ISO 14698, *Biocontamination Control*, in terms of equipment and nutrient medium validation requirements. The latest model of the RCS High Flow (2007) is designed to work with the HYCON-ID System, an integrated process control and electronic data management system for environmental monitoring, which collects and transmits all sample-relevant information to a secure database via the use of data matrix codes and Bluetooth technology (see Figure 5.9).

Gelatin filter sampler: A gelatin filter sampler consists of a vacuum pump that has an extension hose with a filter holder at its end. The filter used for

FIGURE 5.8 Insertion of agar strip in the RCS High Flow. (Photo courtesy of Biotest AG, www.biotest.de/. With permission.)

FIGURE 5.9 The HYCON-ID system. (Photo courtesy of Biotest AG, www.biotest.de/. With permission.)

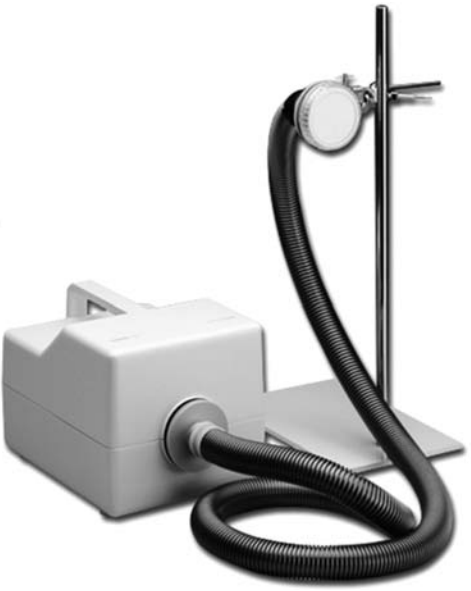

FIGURE 5.10 The Sartorius MD8 air scan. (Photo courtesy of Microbiology International, www.800ezmicro.com. With permission.)

this type of application is composed of random fibers of gelatin capable of retaining airborne microbes. After sample collection is complete, the gelatin filter is aseptically removed, dissolved in a suitable diluent, and processed for bioburden recovery via the pour-plate or membrane filtration method. Following incubation of the test plates at appropriate conditions, recovered colonies are enumerated, and results are reported as number of CFU per volume of air (m^3 or ft^3) sampled. Gelatin filter samplers are ideal for prolonged environmental monitoring of critical areas, without concerns of stressing the gelatin material owing to desiccation [4]. The Sartorius MD8 Air Scan (Microbiology International, www.800ezmicro.com), with gelatin sampling heads 80 mm in diameter, has proven ideal for long-term and remote air sampling during aseptic filling processes (see Figure 5.10).

Active Air Sampling for Nonviable Particles

Testing for nonviable air particulates is performed using electronic particle counters such as the Met-One (Hach Ultra, www.hachultra.com), the LASAIR II (Particle Measuring Systems, www.pmeasuring.com), and the APC M3 (Biotest AG, www.biotest.de/). These units are calibrated laser particle counters designed to sample a defined volume of air that can measure a variety of particle sizes (most commonly 0.5 to 5.0 µm particles). Some are handheld devices, and others have capability for remote and continuous sampling. Results generated are reported as number of particles per volume of air (m^3 or ft^3) sampled. The Biotest APC M3, seen in Figure 5.11, is considered the world's fastest portable particle counter. This device has a flow rate capability of 100 L/min, thus collecting 1 m^3 of air in only 10 min.

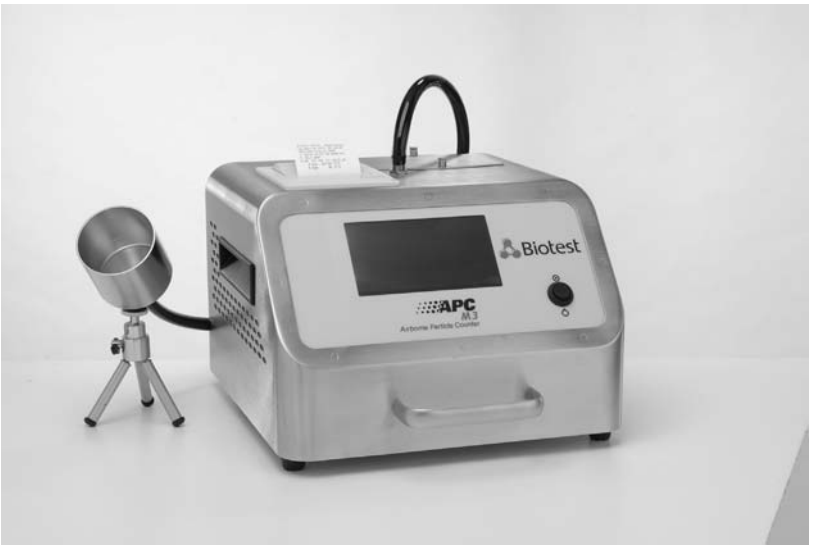

FIGURE 5.11 The APC M3 high-speed portable airborne particle counter. (Photo courtesy of Biotest AG, www.biotest.de/. With permission.)

There are two points to consider in relation to active air samplers. First, when performing EM using remote probes, the user must ensure that the extra tubing does not have an adverse impact on the recovery of viable and nonviable air particulates. In cases where interference is known but cannot be eliminated, the recovered number of particles should be adjusted using a correction factor that can be established during method qualification studies. Second, the user must be aware that microbial recoveries differ depending on the type of equipment and test methodology used; therefore, once an air sampler and test method are chosen and historical data generated, a company cannot change the type of air sampler and/or recovery method without some type of formal evaluation by the company's change control system.

Microbial Identification Program

The microbial identification program developed by a company should be cost effective, scientifically sound, and make good business sense. Identification of every type of microorganism detected is not a regulatory expectation and is certainly an approach that is costly and labor intensive. Microbial identification should be carried out for data trending purposes and to assist product and facility contamination investigations. As discussed in Chapter 4, most companies perform Gram stains of representative colonies when the number of recovered organisms exceeds established alert levels. This simple technique for microbial characterization can serve as a preliminary assessment of potential source of contamination. For example, because most Gram-positive cocci are human borne, high counts of these types of organisms may be indicative of poor gowning/aseptic techniques or improper cleanroom behavior; as most Gram-negative rods are waterborne, and molds thrive in humid

environments, high counts of these types of organisms may indicate wet conditions, standing water, or high humidity levels in the area monitored. Representative organisms from EM monitoring exceeding established action levels are often identified to genus and species levels so that a better assessment of source of contamination and risk to product contamination can be performed. Microbial identification of EM isolates also aids in the evaluation of cleaning/sanitization effectiveness in personnel training and adherence to company procedures, as well as in detecting deterioration and malfunction of systems (e.g., air handing systems) and facilities.

Microbial identification of environmental isolates can be a difficult task for an untrained microbiologist and even for some microbial ID systems on the market. In Chapter 9, the author discusses semiautomated and automated microbial ID systems, including the ones based on genotypic profiles, a method preferred by the FDA [5]. Given the current heightened interest on the part of regulators on environmental monitoring and microbial identification techniques, and given the fact that all microbial ID systems have their strengths and weaknesses, the user must carefully choose the appropriate identification methods that satisfy the objectives of the company's microbial ID program, while being compliant with the current regulatory expectations.

Data Analysis

Evaluation and management of EM data is not an easy task because of the inherent variability and limitations of microbial testing. There is also the need for statistical analysis and trending of EM data, especially microbiological data, because results are retrospective. Prompt evaluation of trended data is critical for a company to detect adverse trends that indicate potential drifts from normal operating conditions. The use of a software program designed for environmental monitoring with the capability of trend analysis is extremely useful as it ensures that key company individuals are notified of excursions in a timely manner. Indeed, prompt and frequent review of EM data is a regulatory expectation [6].

Summary reports containing EM data must be created by the environmental services group and reviewed by Quality and Manufacturing in a timely fashion. EM reports are typically issued on a quarterly and annual basis, and they should include trended data presented in table and graphical formats, as well as representative types of microorganisms isolated. The EM program of controlled environments in pharmaceutical and biotech manufacturing companies is often on the agenda of compliance auditors during inspections. Therefore, EM summary reports provide a platform for companies to present and discuss their microbial control programs and to demonstrate compliance with regulatory expectations in terms of EM data analysis and review.

Traditionally, EM data have been evaluated based on established alert and action levels, as discussed earlier in this chapter and in Chapter 4 of this book. A company must be cautious not to overreact to single excursions that could simply be caused by sampling error or other explainable situations. This is one of the reasons why most companies nowadays prefer to have an EM program that directs management to take action against adverse trends and not single-excursion events.

Another approach being considered and adopted by some companies is the evaluation of EM data in terms of *contamination incidence rate* rather than magnitude of excursions (i.e., alert and action levels). This method is discussed in the article "Environmental monitoring: Data Trending Using a Frequency Model," published in 2004 by the *PDA Journal of Pharmaceutical Science and Technology* [7]. In this document, the authors present two methods to monitor performance of controlled environments where airborne microbial counts are generally zero. Applying EM data with infrequent incidence of microbial recovery, the authors concluded that the methods, *value–moving range chart*, and *exponentially weighted moving average chart* can provide valuable and relevant information in terms of EM data trending.

The main reason for the philosophical change from using numerical values to incident rates is the fact that recent advances in microbiological control have led to less spreads and little statistical significance between alert and action levels calculated based on historical data. In the 2005 draft revision of the USP Chapter <1116>, the USP recommended this shift in paradigm and defined incident rate as the rate at which environmental samples are found to contain any level of contamination. Changes in the incident rate should be viewed as deviations from normal operating/environmental conditions, and therefore, a formal investigation into the event is warranted.

Whenever a company decides to change the method used to trend and react to EM excursions, a new baseline must be established because the new methodology will, most likely, have an impact on existing historical trends. Therefore, prior to implementation, the proposed change should be evaluated via the company change control system.

EM DURING FACILITY VALIDATION ACTIVITIES

Validation of cleanrooms involves activities for design qualification, installation qualification, operation qualification, and performance qualification. During these studies, the air handling systems, room design, and cleaning programs are evaluated.

As discussed earlier in this chapter, the only standards for cleanroom design and certification recognized worldwide are the ISO documents, namely, ISO 14644-1 and ISO 14644-2. These documents define how a cleanroom should perform irrespective of the use and activities in the area. Therefore, EM activities during certification of cleanrooms are carried out under static (at-rest) conditions. However, a company must show that the cleanroom can maintain established air and surface quality standards during production activities; thus, additional EM under dynamic (operational) conditions is performed during performance qualification studies of the manufacturing suites.

As in the case of validation of a new water system, a topic discussed in Chapter 4, during qualification studies of a new or redesigned pharmaceutical suite, a large number of sample sites are chosen and frequently monitored against established industry standards for viable and nonviable contamination. ISO 14644-1 provides a standard for selection of number of testing sites based on room area, but not for testing frequency. Typically, EM monitoring during qualification of a cleanroom is performed daily for a period of 1 week to 30 d. The locations of sampling sites are determined based on industry standards and on a formal assessment of risk of

product contamination. Factors such as equipment location, areas where product is exposed to the environment (open process operations), as well as personnel and equipment flow are taken into account during selection of sampling sites. It is also important to include during this testing phase all the variables, such as sanitization schedules, that will be part of the routine operations of the given cleanroom. Once qualification studies are complete, the cleanroom is released to Manufacturing for production activities, and a reduced EM testing program (frequency and sampling sites) is established for routine environmental monitoring. The data generated are trended, and the information gathered is used as a tool for management to verify the compliant status of the systems in the area over time. This information should also be included in the annual facility review (AFR) report. Based on the evaluation performed, revalidation/requalification studies may be needed, especially in cases where a significant change has taken place.

During the initial phase of monitoring, as well as during routine monitoring, representative environmental isolates are selected for inclusion in the QC laboratory culture collection. These organisms, which represent the typical microbial flora in the manufacturing environment, can be used in disinfectant efficacy studies and in method suitability studies for microbiological examination of pharmaceutical products and materials for pharmaceutical use.

Room Occupancy

As people are the main source of contamination in a manufacturing environment, limiting the number of personnel in a given suite is standard practice during aseptic operations and highly recommended for nonsterile manufacturing operations. Studies to establish personnel room capacity are typically carried out during execution of air handling unit/facility qualification protocols. During these studies, EM is performed for viable and nonviable air particulates over a period of several days under dynamic conditions, with the proposed maximum number of personnel present in the suite. If results obtained are below the set alert levels for the given room classification, the proposed room capacity is deemed suitable for that given manufacturing area. Otherwise, the studies have to be repeated using a reduced number of people in the area.

EM OF ISOLATORS

The focus of EM of isolators is somewhat different from EM of controlled environments because, by definition, an isolator is a leak-proof self-contained environment capable of maintaining aseptic conditions during operations; the main source of contamination, people, is totally eliminated from an isolator system. Isolators are equipped with HEPA filters, and at rest, they meet particulate air quality requirement for a Class 100 environment. However, during operation, an isolator system does not need to meet Class 100 conditions, and there is no requirement for air velocity or air flow laminarity (no turbulence). All materials transferred into an isolator are sterilized via procedures such as vapor-phase hydrogen peroxide (VPHP). The exposure to the sterilizing chemical is carried out in a separate unit attached to the

main isolator, and aseptic manipulations are performed by operators outside the unit wearing flexible half-suits with gloves.

During the validation activities for an isolator system, nonviable particulate monitoring is performed to verify that the unit meets Class 100 standards. Microbiological monitoring is performed as part of the routine EM program to ensure that the isolator is able to maintain an aseptic environment and to detect malfunctions with the sterilization and HEPA filtration systems. The methods used for microbial testing include contact plates for flat surfaces and swabs for irregular surfaces. As discussed earlier in this chapter, the operator must ensure that sites sampled with contact plates are wiped clean with sterile 70% alcohol in order to remove media residue. Active air samplers and/or settling plates or settling broths (e.g., TSB) may also be used during operations. Regardless of the microbial recovery method chosen to monitor aseptic condition during operations, one must ensure that the EM testing device is not intrusive so that it does not compromise the aseptic conditions of the isolator environment.

Since the introduction of isolators to the pharmaceutical industry, the extensive amount of data collected have indicated that these systems are not completely impervious to microbial contamination, and therefore, their sterility conditions may be compromised. The two most likely sources of microbial contamination are defective soft parts (e.g., gaskets, half suits, and gloves) and improper decontamination of materials and supplies. VPHP decontamination requires that surfaces be exposed to gaseous sterilants for a given period of time. If materials are not laid out properly in the decontamination/transfer unit, the VPHP will not be able to come in contact with all the materials' surfaces, thus potentially leading to introduction of bioburden into the main isolator unit. A company must understand and know these limitations to isolation technology and incorporate in its EM program surface monitoring of sterilized materials, periodic inspection of gaskets and other soft parts, and routine testing to detect small leaks in gloves and half suits. There are commercially available glove leak detectors that can be used as part of a routine maintenance program for isolators. However, in addition to physical tests for leak detection, the USP recommends in Chapter <1208>, *Sterility Testing—Validation of Isolator Systems*, microbiological testing of gloves by direct immersion to detect low-level microbial contamination that may not be detected with contact plates or swab samples. This can be accomplished by submerging the gloves in 0.1% peptone water, followed by membrane filtration and plating on growth medium. The USP also recommends routine monitoring for nonviable particulates (continuous monitoring is preferred) so that a company can detect filter failure in a proactive and timely manner.

There is no set standard for the frequency of EM monitoring of isolators. As a general rule, isolation systems should be monitored following routine sterilization and on the last day prior to the next surface decontamination cycle. Most companies also perform some type of microbiological testing during operations and routinely at the end of operations.

MICROBIAL CONTROL IN CLEANROOMS

In order to minimize microbial contamination, a company must minimize the number of particles in the area. It is important to note that, to date, data generated in

the pharmaceutical industry provide no scientific agreement on the relationship between the number of nonviable particulates and the bioburden in the environment. However, because microorganisms become airborne and are transported via shed particulates, controlling nonviable particulates in cleanrooms is essential to ensure microbial control.

As discussed earlier, the main source of microbial contamination in a pharmaceutical manufacturing environment is humans—people generate both viable and nonviable particles even when wearing a two-piece coverall. Particulates shed by personnel originate from exposed skin and hair, clothing, perspiration, as well as oral emissions (e.g., from talking, coughing, and sneezing). In order to minimize human-borne contamination, companies should enforce proper cleanroom behavior and attire—no exposed jewelry and no makeup, and gowning must be of proper size and type to minimize exposed skin and hair. A company must also have procedures to prevent sick personnel from working in manufacturing areas.

There are other sources of microbial contamination besides personnel, and these include poor cleanroom and equipment designs, and nondurable materials of construction. Companies must ensure that the finishing materials on ceilings, walls, and floors will withstand routine traffic and room sanitization procedures. The room design must ensure that light fixtures, doors, and windows are flush with the inside of the cleanroom, HEPA filters are appropriately located and installed, and the floor slopes toward the drain. In addition, equipment must be of sanitary design and appropriately located to permit easy access for maintenance and room cleaning, and must not block air return vents. The air supplied from the ceiling should flow in a nonaspirating manner to reduce air turbulence, especially above open-process operations. Other factors that should be considered and controlled for prevention of microbial contamination include room temperature (ideally 18–21°C), relative humidity (ideally 30–60%), and air-pressure differential (positive pressure in relation to adjacent and less clean area). A positive pressure differential of 10–15 Pa between a cleanroom and an adjacent room of lower classification complies with the U.S. and European cGMP standards. The exception is the use of negative pressure in cleanrooms designed to provide biocontainment and where flammables or potent chemicals are handled.

Microbial contamination is also controlled via effective sanitization practices, good housekeeping, and aseptic technique of operators, which must be governed by company standard operating procedures and enforced by management.

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6 Bioburden Considerations in Equipment-Cleaning Validation

Equipment used in pharmaceutical and biopharmaceutical production is vulnerable to varying degrees of microbial contamination that originate from many sources. An ineffective equipment-cleaning program can have many negative consequences, including the risk to patient health and quality of product manufactured that could in turn lead to product withdrawal from the market. It is therefore not surprising that cleaning validation undergoes extensive regulatory review in the pharmaceutical industry during inspections. In fact, during the past few years, cleaning validation has ranked among the top 10 areas of concern in warning letters issued by the FDA. According to Kristen Evans, leader of the Guidance and Policy Team in the Division of Manufacturing and Product Quality in FDA's Center for Drug Evaluation and Research, equipment cleaning and maintenance ranked second in warning letters and GMP citations for the fiscal year (FY) 2004–2005 [1]. Although not all cleaning issues relate to microbial contamination, controlling bioburden through adequate cleaning processes is a regulatory expectation enforced by the FDA as illustrated in the following excerpts from two regulatory citations:

- FDA 483 issued to Evans Vaccines (Merseyside, UK), an affiliate of Chiron Corporation, regarding product sterility failure investigation in October 2004: "Cleaning validation for the CIP process for vessel … which is utilized in the aseptic formulation of trivalent bulk influenza, did not include an assessment of sprayball coverage for the vessel. In addition, the study *did not include swab sampling of the transfer lines* used in the transfer of monovalent blend pools into the mixing vessel … and for transferring the aseptic trivalent formulated bulk back into a sterilized … liter tank in formulation room."
- FDA warning letter issued to MedImmune Inc. (Gaithersburg, MD) on May 24, 2007, regarding bioburden deviations in the manufacture of bulk

> monovalent lots for FluMist vaccine during the 2006–2007 campaign: "Of particular concern are your inadequate investigations into such excursions, and your lack of implementation of appropriate corrective and preventative actions, coupled with *deficiencies in*: aseptic practices by personnel, *cleaning validation of equipment* and *effectiveness of the cleaning and disinfection processes* used in your manufacturing facility and by your personnel."

Microbial contamination is a multiparametric process, which is affected not only by sources of contamination but also by the environmental conditions and properties of contaminated materials (e.g., surface charge, hydrophobicity, texture, etc.), which can contribute to microbial adhesion and proliferation. Although sterilization and sanitization procedures for equipment are beyond the scope of the FDA *Guide to Inspections—Validation of Cleaning Processes*, the FDA does address in this document concerns regarding control of bioburden in process equipment. Indeed, the FDA expects companies to have written procedures not only to prevent ingress of contamination but also to eradicate or reduce bioburden through validated sterilization and cleaning procedures. Ideally, production equipment used in the manufacture of drugs that will be rendered sterile should be sterilized (e.g., steamed in place or autoclaved) to prevent product contamination.

However, due to the equipment design and materials used in construction, many large systems such as chromatography skids and columns cannot be sterilized, and therefore must be cleaned in place using chemical sanitizer solutions. In addition, although equipment used in nonsterile manufacturing is not required to be sterile, bioload on equipment surfaces must be reduced to acceptable levels that will ensure product quality and safety. In the *Guide to Inspections—Validation of Cleaning Processes*, the FDA states that whether or not clean-in-place (CIP) systems are used for cleaning of processing equipment, microbiological considerations should be given to equipment-cleaning protocols through either preventative measures or removal of contaminants. The FDA also indicates that companies should provide evidence that routine cleaning and storage of equipment do not allow microbial proliferation, issues also addressed in the regulatory guidance documents: *Good Manufacturing Practices Guidelines* # 02-122102-681 [2] and the Pharmaceutical Inspection Convention/Pharmaceutical Inspection Cooperation Scheme (PIC/S) document PE 009-1 [3].

In this chapter, the main regulatory concerns and expectations in terms of bioburden and endotoxin as it relates to equipment-cleaning validation are discussed. Although the topics of sterilization and chemical residues (e.g., total organic carbon [TOC]) are beyond the scope of this book, readers are encouraged to become familiar with sterilization processes for pharmaceutical manufacturing equipment and TOC testing for verification of equipment cleaning along with the applicable regulatory requirements and guidance documents.

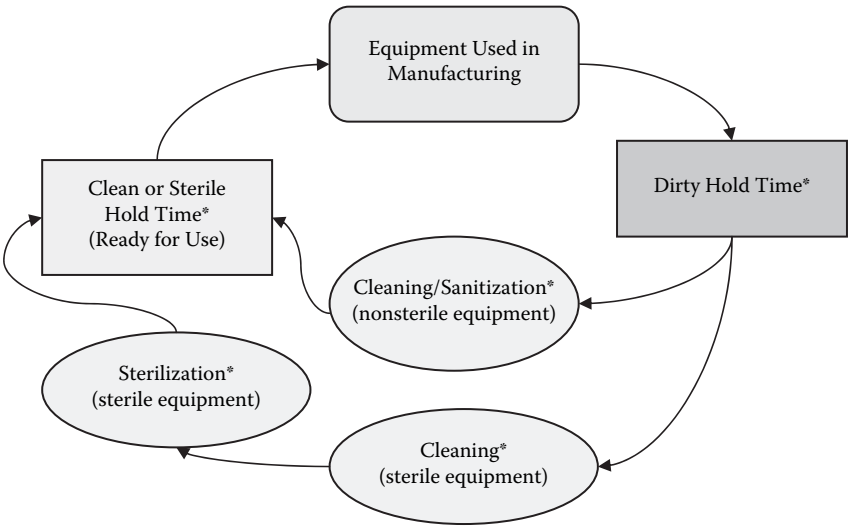

FIGURE 6.1 Clean-and-use cycle of manufacturing equipment.

BIOCONTAMINATION CONTROL

Contamination can be defined as the presence of a substance (e.g., solid, liquid, gaseous, biological, or chemical) that is likely to negatively affect the safety or quality of the product manufactured. Regarding microbial contaminants, risk considerations include not only viable organisms detected by bioburden recovery methods but also their residues, such as endotoxins, enterotoxins, and proteases. For equipment that are not required to be sterile, cleanliness is not absolute. Acceptable levels of bioburden and bacterial endotoxins (e.g., applicable to equipment used in the manufacture of parenteral and sterile nonpyrogenic inhalation products) are established by the manufacturer based on the types of processes (e.g., dry formulation, bioprocess, sterile), step in the process (e.g., upstream, downstream), and whether equipment can be steamed in place or is chemically sanitized.

The *clean-and-use cycle* of pharmaceutical manufacturing equipment has various stages that include validated processes for cleaning/sterilization of equipment and validated hold steps (Figure 6.1). For a risk-based approach to validation of cleaning processes, the greatest risks to microbial contamination of production equipment are from initial ingress and storage (*clean-hold time*). *Clean hold* is defined as a state in which the equipment is ready for use. Consideration should also be given to the possibility of microbial proliferation during equipment *dirty-hold time*, which is defined as the maximum qualified amount of time that the equipment can remain soiled prior to cleaning.

Microbial contamination can be introduced into a piece of equipment (initial ingress) via raw materials and excipients, during open connections as well as during changeover procedures. In most cases, effective cleaning prior to use ensures microbial control through the elimination or reduction of bioburden introduced into

the equipment. However, bioburden can proliferate and form biofilms if the sanitization and equipment storage solutions are not effective, and whenever the equipment is not of sanitary design (e.g., with dead legs, inappropriate finishing materials, not free draining, and with crevices). Indeed, microbiological cleanliness is attained not only through control of bioburden ingress and equipment sanitization procedures but also through

- Assurance of antimicrobial effectiveness of sanitization and storage solutions
- Use of sanitary equipment as specified in the 3-A Sanitary Standards (www.3-a.org)
- Maintenance of clean conditions during clean-hold time
- Storage of equipment in dry conditions after cleaning whenever possible
- Use of cold storage for chromatography resins or packed columns and other similar equipment/materials
- Extensive operator training and supervision to ensure strict adherence to cGMPs procedures
- Cleaning the equipment as soon as possible following use
- Sanitizing cleaned equipment prior to use

Disposable and Single-Use Equipment

In the 21st century, many single-use and disposable pieces of equipment and materials have been introduced in pharmaceutical manufacturing. In biopharmaceutical production, disposable flasks, filtration systems, spinner bottles, and even bioreactors have been used. The main advantages to using disposable equipment are of course prevention of microbial contamination and elimination of the need for cleaning validation and its associated costs. Indeed, the use of disposable/single-use equipment and materials is a trend in the pharmaceutical industry, and the regulatory agencies are openly endorsing these technologies. However, there are still some issues that must be addressed if a company chooses to employ single-use equipment, such as scalability, compatibility with raw materials and products, the need for preuse cleaning/flushing, and concern with extractables (compounds that migrate from material into solvents under extreme temperature and time exposure) and leachables (compounds that migrate into the drug product under normal processing conditions). Despite some of these concerns and, perhaps, some challenges posed by the possible unfamiliarity of regulatory inspectors with single-use technologies, the use of disposable materials and equipment does and will provide great benefits to pharmaceutical and biopharmaceutical companies in terms of biocontamination control and prevention. Impact, if any, of both leachables and extractables from disposable equipment can be addressed during process validation, and when carefully evaluated, it is found that they rarely have a negative impact on the quality and safety of products, and so their use is easily defendable.

Equipment-Cleaning Methods

Equipment-cleaning methods use chemical and/or physical means and they fall into two main categories: clean-in-place (CIP) or steam-in-place (SIP), and clean-out-of-

place (manual). For chemical cleaning, CIP is preferred because it can be automated and the equipment need not be disassembled. CIP systems, which can either be hard piped to the production vessels or used as a mobile unit, are able to clean equipment in situ. For sterilization of large pieces of equipment, such as production vessels, companies use SIP cycles, whereas small pieces of equipment and materials are autoclaved. Regardless of the type of method used, a validation study must be performed to ensure the effectiveness and reproducibility of the cleaning/sterilization procedures. This chapter will focus on the cleaning and sanitization of equipment that are not required to be sterilized.

Chemical cleaning can be achieved via oxidation, hydrolysis, and enzymatic action. Effective cleaning and sanitization procedures include, but are not limited to, the following:

- Treatment with acids and bases
- High-velocity hot water, steam, solvent rinses
- Use of sanitizers and detergents
- Drying at high temperatures

The quality of the water used in the preparation of cleaning solutions and as a prerinse or postrinse is critical. In general, for manufacturing nonsterile products, the use of purified water to clean and rinse production equipment is suitable. For cleaning and rinsing equipment used in the production of sterile drugs and in downstream processing (purification steps in biopharmaceutical production), the water used must meet the water for injection (WFI) quality attributes.

In biopharmaceutical manufacturing, maintaining nonsterile equipment (e.g., packed columns) under microbial control is a challenge. Alkaline solutions, such as sodium hydroxide (from 0.1 to 1.0 M solutions), are widely used as cleaning agents and for regeneration and storage of chromatography media (carbohydrate and polymeric matrices) because alkaline solutions are particularly effective in removing proteins and fermentation process residues. A 1 M sodium hydroxide (NaOH) solution is a good sanitizer and capable of reducing, but not totally inactivating, spores of *Bacillus subtilis* when used at 22°C [4]. NaOH is also very effective in removing fungi and endotoxins. Some cleaning solutions are supplemented with sodium hypochlorite (about 400 ppm), salt (e.g., sodium chloride), or alcohol to combine cleaning with sanitization. Although sodium hypochlorite is a well-known sanitizer, its use is somewhat limited due to risk of damage to materials, the possibility of generating toxic chlorinated by-products, and its impact on the chemical stability of chromatography resins.

Peracetic acid, a strong oxidizer with sporicidal properties, offers some advantages over sodium hypochlorite for chromatography column cleaning. Studies performed indicated that a 22°C solution of peracetic acid (1500 ppm) supplemented with 30% ethanol and in 0.5 M acetate buffer, at pH 5, showed a 90% reduction (D_{10} value) in a population of *B. subtilis* spores within 0.4 min compared to a D_{10} value of about 860 min obtained for a 0.5 M NaOH solution at 4°C [5]. In addition, there are safety benefits to the use of peracetic acid as this compound decomposes to acetic acid, oxygen, and water. Acid–cleaner formulations combine at least one

acid and a surfactant. These chemicals are effective in removing alkaline salt, some sugars, and particulate matter residues. Enzymatic cleaners are substrate specific and accelerate the breakdown of certain organic residues. Hydrolysis renders some types of chemical residue soluble in water and helps their removal from surfaces via a chemical reaction of an aqueous alkaline or acidic solution with an ester, ether, or amide compound.

When selecting a cleaning agent, a company must evaluate its chemical compatibility with the equipment and materials surfaces, ecological compatibility, chemical toxicity, and contact time required. Other factors that must be taken into account while selecting a cleaning method include

- Static immersion versus dynamic flow
- Need for mechanical cleaning
- Temperature of the cleaning agent
- Volume of the cleaning solution (e.g., from 1 to 10 column volumes)

Selection of a cleaning agent is critical because the chemical it contains is considered a contaminant to the process, and its removal and possible interference with bioburden recovery must also be demonstrated during cleaning validation studies.

VALIDATION OF CLEANING METHODS

Validation of cleaning methods is required to ensure that the equipment-cleaning cycle consistently provides results that meet acceptable levels of cleanliness. Guidelines for cleaning requirements are provided to the pharmaceutical industry in the cGMP documents in the United States, Europe, and other countries. Equipment-cleaning requirements are also addressed in the PIC recommendations and the PDA Technical Report No. 29, *Points to Consider for Cleaning Validation* [6]. Cleaning validation for microbial contamination and endotoxin residues involves testing equipment surfaces to ensure that the cleaning methods reduce contamination to preestablished acceptable levels. For bioburden analysis, validation is also needed to establish an adequate storage condition for the dirty (dirty-hold time) and cleaned (clean-hold time) equipment.

The first step in establishing a cleaning validation program is to define the strategy to be used to ensure the efficacy and reproducibility of the cleaning procedures. Typically, a company starts the qualification studies with selected neutral agents (e.g., hot water at high velocity) or with chemicals known to be least harmful to the equipment. If initial test results fail, then other cleaning agents are evaluated.

Cleaning validation should be implemented via a validation master plan that can be drafted using guidelines provided in various regulatory documents for pharmaceutical production, such as the ICH Q7, *Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients*. As part of a cleaning validation master plan, a company selects the sampling and detection methods, sets the acceptable levels of contamination prior to protocol approval, executes the protocol, collects and analyzes data, and writes procedures for cleaning verification and the training

of operators who clean the equipment. For manual cleaning methods, qualification/validation of personnel must also be performed to demonstrate reproducibility in cleaning results.

Some of the topics that address cleaning validation and are highlighted in the ICH Q7 document include

- Cleaning validation should be performed for process steps in which contamination or material carryover poses the greatest risk to product quality.
- Cleaning validation should reflect actual patterns for equipment usage.
- A company should use validated methods that have the sensitivity to detect residues and contaminants.
- Equipment cleaning/sanitization studies should address microbiological and endotoxin contaminations, as appropriate.
- Cleaning validation should include monitoring of equipment at appropriate intervals to ensure that cleaning procedures are effective during routine production.
- Sampling should include swabbing, rinsing, or alternate methods, as appropriate.

Sampling Recovery Methods

One of the main steps in equipment-cleaning validation is selecting the best residue detection method. There are two primary types of sampling techniques widely used in cleaning validation studies and during the routine monitoring of pharmaceutical equipment and surfaces: *direct surface sampling* (*swabbing)* and *rinsing* (diluent or placebo). There are advantages and disadvantages to both techniques as described in Table 6.1. Many cleaning verification protocols combine both techniques, depending on the type of equipment that needs to be sampled.

For bioburden recovery in cleaning validation studies, the focus is on recovery of mesophilic aerobic microbes. For this purpose, TSA medium incubated at 30–35°C is suitable. However, alternate media and incubation conditions may be required if the detection of a specified microbial species is a concern.

Bacterial endotoxins are typically detected from swab and rinse samples using the Limulus amebocyte lysate (LAL) method. If swabs are used, extraction methods must be developed prior to processing the samples.

Swabbing of Equipment

Equipment swabbing must be performed by qualified personnel, and sterile swabs made from materials that do not interfere with the test should be used. There are various types of swabs used to monitor flat or hard-to-reach surfaces such as the bottom of a tank, O-rings, traps, transfer lines, and U-bends. As mentioned in Chapter 5, the QUANTISWAB® (bioMérieux Inc., Hazelwood, MO) has been proven an excellent choice for bioburden recovery from surfaces. Swab sampling should be carried out wearing sterile gloves to minimize adventitious contamination. For bioburden recovery, after swabbing is complete, the swab may be streaked onto an agar medium or broken into a neutralizing diluent, vortexed for about 30

TABLE 6.1
Comparison between the Two Primary Types of Sampling Techniques: Swabbing and Rinsing

Swabbing		Rinsing	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Sampling of hard-to-reach areas	Some swabs may trap microorganisms and reduce recovery	Ability to sample large surface area	Rinse solvent may not dislodge bioburden that has adhered to the surface
Sampling of defined surface area	Technique/operator dependent	Sampling of areas not accessible by swabs	Water can lead to cell lysis and reduced microbial recovery
Adds capability of physical removal	May lead to adventitious contamination during sampling	Ability to automate (online monitoring) and contain (less exposure to environment)	Large dilution may lower test sensitivity
Economical	Invasive technique	Less intrusive/no need to disassemble equipment	Inability to determine the exact location of contamination
Widely available	Results subject to site selection/assumes uniform contamination	Technique independent as compared to swabs	Difficult to control the area sampled

s, and the liquid sample preparation tested by pour-plate or membrane filtration method. The incubation conditions for the recovery media vary depending on company protocols. However, in general, swab preparations are plated with TSA and plates incubated at 30–35°C for 3–5 d. Results are reported as number of CFU per swab or area sampled. If swabs are to be transported to the testing laboratory, they need to be stored in a manner that preserves the samples collected as well as prevents adventitious contamination.

Rinsing of Equipment

Equipment rinse is performed using a solvent that will not interfere with recovery of residues. Sometimes placebo is used, although this approach is not typical for collection of bioburden or bacterial endotoxin. Most rinse samples are collected using purified water or WFI. For bioburden recovery, after the rinse sample is collected, it is processed via the membrane filtration technique, the filter plated onto TSA, and the agar plate incubated at 30–35°C for 3–5 d. The use of water for equipment rinse may interfere with microbial recovery due to cell lysis. Although this topic is not addressed in cleaning validation articles and reference documents, a company should include test controls in the validation recovery studies to assess the possibility of low bioburden recovery due to loss of microbial viability. Also, rinsing and swabbing (to a certain extent) are only partially effective in removing cells from a multilayer biofilm. Companies must consider this fact when analyzing equipment-cleaning data because microbial recovery methods may only provide a semiquantitative indication of the microbial contamination on equipment surfaces.

Qualification of Sampling Methods

Part of a cleaning validation study and typically the first protocol step executed is the actual qualification of the test methodologies chosen to assess the microbiological quality of equipment surfaces. One must demonstrate that the chosen method is able to recover viable organisms and that any product or cleaning agent residue will not interfere with their detection. One key point to consider is that the limit of quantitation (LOQ) for the method developed must be equal to or below the established cleaning validation limit for the given piece of equipment. Although not discussed in detail in this chapter, the same principles apply to qualification of methods to detect bacterial endotoxin on surfaces. However, due to difficulties in recovering bacterial endotoxin from surfaces using swabs, rinsing is often the method of choice.

Qualification of recovery methods is performed using clean and sterilized/sanitized coupons, which are small pieces of material representing the equipment to be sampled (e.g., stainless steel, polypropylene, glass, silicone, etc.). The use of coupons avoids the contamination of the actual equipment or manufacturing environment.

Bioburden recovery is determined as a percentage of the original amount of microbial inoculum added to the coupons. This value can be affected by many factors such as the inherent variability of microbial recovery methods, desiccation of vegetative cells, the type of swab used, the sampling technique, and the type of equipment surface (regular versus irregular). Therefore, in order to ensure the correctness of test results during assessment of equipment cleaning, a study to determine the accuracy and reproducibility of the chosen recovery methods is required.

A bioburden recovery test is performed by inoculating sterile/sanitized coupons with a known level of microorganisms (typically less than 100 CFU per coupon) and sampling the surface as proposed in the cleaning validation protocol. The number of recovered organisms is compared to the number of inoculated organisms and a percent recovery rate is calculated. For chemical residues (including endotoxin), a recovery greater than 80% is considered good, a recovery greater than 50% is considered reasonable, and a recovery value less than 50% is considered questionable [7]. However, for bioburden, there is no set standard for an acceptable recovery from surface sampling; the USP Chapter <1227> *Validation of Microbial Recovery from Pharmacopeial Articles* is designed to show adequate neutralization for testing of microbial limits in pharmaceutical products and raw materials, and does not apply to bioburden surface recovery methods. In general, a standard practice in the industry, and one recommended by the author, is to establish a minimum bioburden recovery of 50%, a value that has been found acceptable by most regulatory investigators.

The test organisms often used to inoculate the coupons represent a wide spectrum of microbial flora typically found in a manufacturing environment and that may be detrimental to pharmaceutical equipment and products (see Table 6.2). Other isolates, including environmental strains, can be added to the test organism panel or as a substitute for the American Type Culture Collection (ATCC) species at the discretion of the manufacturer. Organism suspensions can be prepared as described in Chapter 7, or used directly from commercial preparations that have a predetermined population range.

TABLE 6.2
List of Test Organisms

Organism Name	ATCC Number	Organism Type
Staphylococcus aureus	6538	Gram-positive coccus
Bacillus subtilis	6633	Gram-positive spore-forming rod
Pseudomonas aeruginosa	9027	Nonfermenting, Gram-negative rod
Escherichia coli	8739	Fermenting, Gram-negative rod
Candida albicans	10231	Yeast
Aspergillus niger	16404	Filamentous fungus
Environmental isolates (company specific)		
Others as needed (e.g., specified microbial species)		

Recovery Study Using the Wet Method

This procedure is performed for vegetative cells (bacteria and fungi) to prevent loss of viability due to desiccation (Figures 6.2 and 6.3). Inoculate each type of coupon with less than 100 CFU of the inoculum suspension prepared, for example, in 0.1% peptone water. Ideally, the inoculum level should be between 25–100 CFU. An inoculum with less than 25 CFU may lead to greater than normal plate count variability,

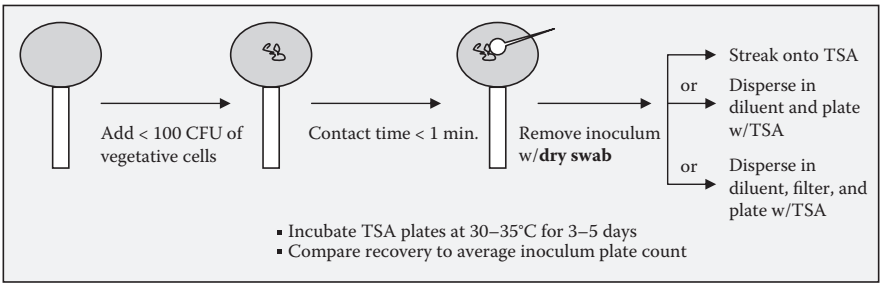

FIGURE 6.2 Swab recovery qualification using the wet method.

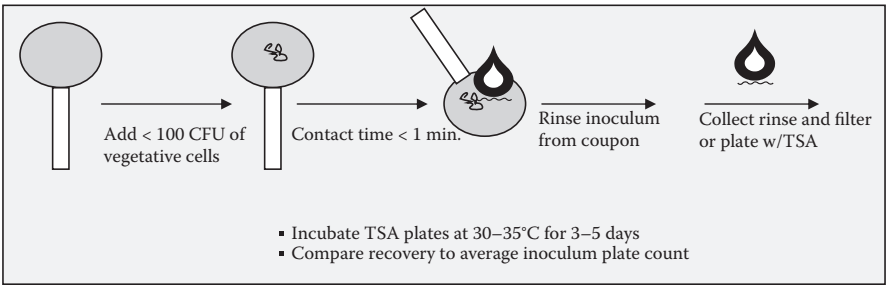

FIGURE 6.3 Rinse recovery qualification using the wet method.

thereby reducing the accuracy of the test. An inoculum count greater than 100 CFU may not be considered a low-level inoculum; besides, it may lead to reduced accuracy during plate counting due to crowding effects.

Verify the inoculum level by the pour-plate or streak-plate method, in duplicate or triplicate, using TSA medium and incubating test plates at 30–35°C for a minimum of 2–3 d. After the liquid inoculum is added to the coupon surface, allow a contact time of less than 1 min. Use either the swab or rinse method to recover the test organisms:

- Swab method: Using a dry swab, remove the liquid inoculum from the surface of the coupon. Streak the swab onto an agar medium or break it into a neutralizing diluent, vortex for about 30 s, and process the liquid sample preparation by either pour-plate or membrane filtration method. Use TSA with incubation conditions at 30–35°C for 3–5 d. Results are reported as number of CFU per swab.
- Rinse method: Place the inoculated coupon in an aliquot of sterile diluent (e.g., purified water) contained in a sterile vessel, which will be used to rinse the given piece of equipment. Shake well and then remove the coupon aseptically. Attempts should be made to best mimic the rinse solution temperature, contact times, and cleaning conditions (e.g., dynamic flow versus static contact). Filter the rinse solution through a 0.45-µm membrane filter. Then, aseptically remove the membrane filter and place it onto a solidified TSA agar plate. Incubate the prepared plates at 30–35°C for 3–5 d. At the end of the incubation period, enumerate the recovered colonies and report results.

Perform this procedure (swab and/or rinse sampling), in triplicate, for each challenge organism. Perform a test-negative control for each swab and rinse set to verify aseptic manipulations by carrying out the procedure just described but with uninoculated coupons.

Average the counts obtained for the three swab and/or rinse sample preparations and compare results to the average result obtained for respective inoculum count plates. Calculate the percent recovery for each test organism. For example,

Average count for inoculated coupons: 35 CFU
Average count for inoculum level: 46 CFU
Percent recovery: (35 CFU/46 CFU) × 100 = 76%

Test acceptance criteria

- If values are below the established acceptance criterion (e.g., less than 50%), the cause for the less-than-adequate recovery should be investigated and eliminated prior to performing any retests. Modifications to the swab/rinse technique to improve microbial recovery may include the use of an alternate type of swab, or use of an alternate recovery method and/or medium.

- If any of the inoculum level counts exceeds 100 CFU, the test should be repeated.
- If microbial growth is recovered from the negative control samples, the test should be repeated.

Recovery Studies Using the Dry Method

The *dry method* is to be used for spore-forming bacteria only because vegetative cells suffer desiccation and, therefore, would not be viable on dry surfaces (Figures 6.4 and 6.5). Inoculate each type of coupon with less than 100 CFU of the spore preparation inoculum suspension. Allow the inoculum to evaporate to dryness under laminar flow conditions.

Verify the spore inoculum level used by the pour-plate or streak-plate method, in duplicate or triplicate, using TSA medium and incubating test plates at 30–35°C for a minimum of 2–3 d. After the inoculum is added to the coupon surface and dried, use either the swab or rinse method to recover the test organisms:

- Swab method: Using a wet swab, remove the dried inoculum from the surface of the coupon. Streak the swab onto an agar medium or break it into a neutralizing diluent, vortex for about 30 s, and process the liquid sample preparation by either pour-plate or membrane filtration method. Use TSA with incubation conditions at 30–35°C for 3–5 d. Results are reported as number of CFU per swab.

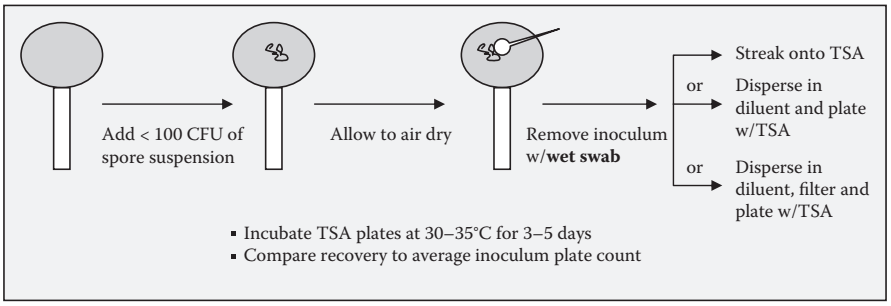

FIGURE 6.4 Swab recovery qualification using the dry method.

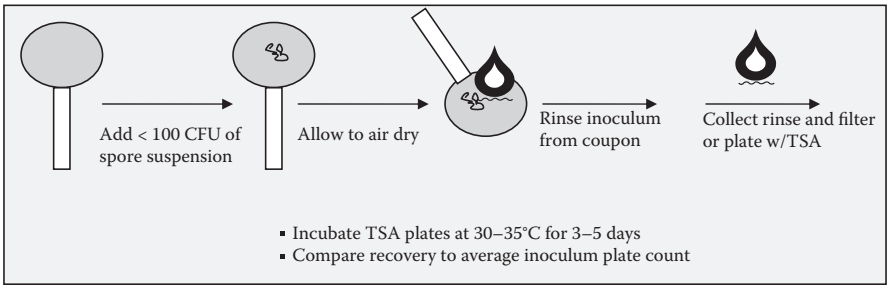

FIGURE 6.5 Rinse recovery qualification using the dry method.

- Rinse method: Place the inoculated coupon in an aliquot of sterile diluent (e.g., purified water) contained in a sterile vessel, which will be used to rinse the given piece of equipment. Shake well and then remove the coupon aseptically. Attempts should be made to best mimic the rinse solution temperature, contact times, and cleaning conditions (e.g., dynamic flow versus static contact). Filter the rinse solution through a 0.45-μm membrane filter. Then, aseptically remove the filter and place it onto a TSA agar plate. Incubate the prepared TSA plates at 30–35°C for 3–5 d. At the end of the incubation period, enumerate the recovered colonies and report results.

Perform this procedure (swab and/or rinse sampling), in triplicate, for each challenge spore-forming bacterium. Perform a test-negative control for each swab and rinse set to verify aseptic manipulations by carrying out the procedure just described but with uninoculated coupons.

Average the counts obtained for the three swab and rinse sample preparations and compare the results to the average of the result obtained for the respective inoculum count plates. Calculate the percent recovery for each test organism as described earlier in this chapter.

Test acceptance criteria

- If values are below the established acceptance criterion (e.g., less than 50%), the cause for the less-than-adequate recovery should be investigated and eliminated prior to performing any retests. Modifications to the swab/rinse technique to improve microbial recovery may include the use of an alternate type of swab, or use of an alternate recovery method and/or medium.
- If any of the inoculum level counts exceeds 100 CFU, the test should be repeated.
- If microbial growth is recovered from the negative control samples, the test should be repeated.

Effects of Product and/or Cleaning Agent Residue on the Recovery of Microorganisms

The purpose of this qualification study is to evaluate any interference from product and/or cleaning agent residue on the recovery of microorganisms (Figures 6.6 and 6.7). As such, this study demonstrates that the chosen medium/diluent ensures adequate neutralization of any chemical inhibitory effects. This study should be performed prior to the execution of the cleaning validation protocol and after the determination of a suitable recovery method.

In order to determine whether product residue might interfere with the recovery of test organisms, a solution of the product is prepared (in an appropriate sterile diluent) at the maximum allowable concentration level for a residue left on the equipment surface after the cleaning procedure. Coupons are immersed in and coated with the prepared product dilution, aseptically removed, and allowed to dry under laminar flow conditions. In order to evaluate any interference from the cleaning agent on

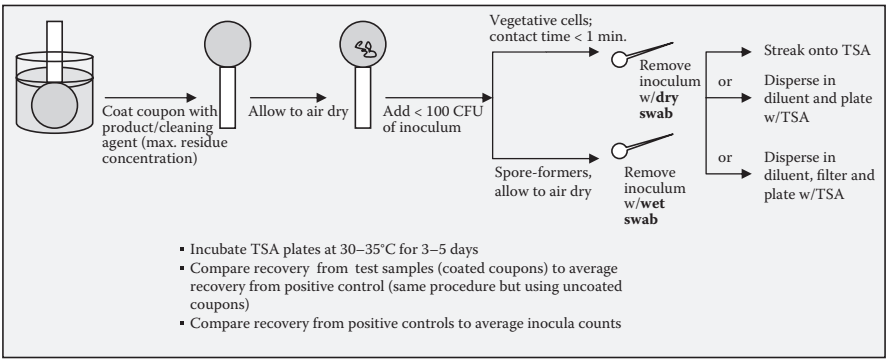

FIGURE 6.6 Product/cleaning agent residue interference study using the swab method.

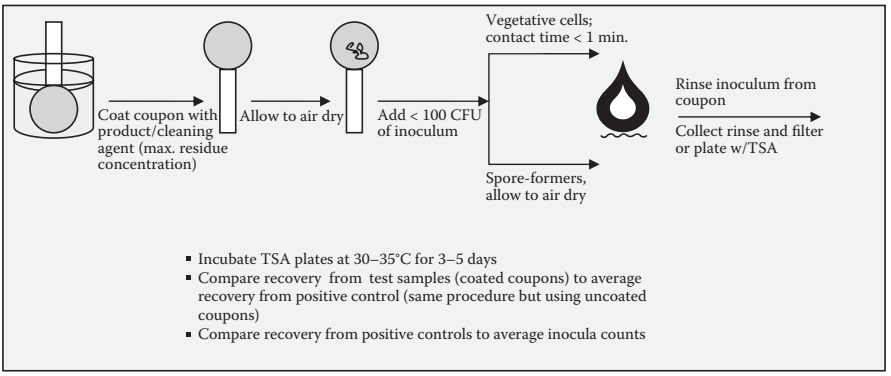

FIGURE 6.7 Product/cleaning agent residue interference study using the rinse method.

microbial recovery, a separate set of coupons is immersed and coated with a solution of the cleaning/sanitizer agent prepared at the maximum allowable concentration level for a residue left on the equipment surface after the cleaning procedure. In case it is known or suspected that the combined product/cleaning agent residue may have an additional adverse effect in the recovery of microorganisms, it is recommended that the test be performed using a mixture of the two solutions.

After the coupons are coated with product or cleaning solution and dried, each coupon is inoculated with less than 100 CFU of each of the test organisms. Perform the procedure, in triplicate, for each type of coupon and challenge organism. For vegetative cells, process the test preparations within 1 min of inoculation. For spore-forming bacteria, allow inocula to dry under laminar flow conditions.

Prepare test-positive controls by inoculating untreated (uncoated) coupons with less than 100 CFU of each of the test organisms, as described earlier in this chapter. Verify the inoculum level by the pour-plate or streak-plate method, in duplicate or triplicate, using the TSA medium and incubating test plates at 30–35°C for a minimum of 2–3 d. Perform a test-negative control for each swab and rinse set to verify aseptic manipulations by carrying out the procedure just described but using uninoculated and uncoated coupons.

To recover the microorganisms from the test coupons (coated) and positive control coupons (uncoated), use the swab and/or rinse methods by either the wet method or dry method approaches, as applicable. Depending on the type of product and cleaning agent used, addition of appropriate and specific chemical neutralizers to the recovery medium and/or rinse solution may be required. Test plates are incubated at 30–35°C for 3–5 d. At the end of the incubation period, enumerate the recovered colonies and report results. Average the counts obtained for the three swab and rinse product/cleaning agent–coated coupon preparations, and compare the results to the average counts obtained for the respective test-positive controls (uncoated coupons). Calculate the percent recovery for each test organism.

Test acceptance criteria

- If values are below the established acceptance criterion (e.g., less than 50%), the cause for the less-than-adequate recovery should be investigated and eliminated prior to performing any retests. The use of an alternate recovery medium, to include the use of appropriate chemical neutralizers, and/or incubation conditions may be required to reduce product/cleaning agent interference in microbial recovery.
- If any of the inoculum level counts exceeds 100 CFU, the test should be repeated.
- If microbial growth is recovered from the negative control samples, the test should be repeated.

Establishing Limits

As discussed earlier in this chapter, acceptance criteria for bioburden and endotoxin should be calculated and justified as a function of the nature of the product manufactured and the stage of the process in which equipment is used (e.g., upstream versus downstream processing). For bioburden, in addition to numerical values, requirement for absence of given microbial species may be needed based on the type of product that comes in contact with the equipment. According to the FDA *Guide to Inspections—Validation of Cleaning Processes*, it is impractical for the FDA to set acceptance specifications or methods for determining whether a cleaning process is valid or not. However, the FDA states that limits for cleaning validation should be logical, practical, achievable, verifiable, and should be based on scientifically sound methodologies. There are a few guidance documents that provide recommendations for establishing limits for chemical residues. *The Cleaning Validation Guidelines* published by Health Canada (last revised June 18, 2002) is one such document. As far as chemical residue is concerned, it is standard industry practice to accept the following limits as proof of validation of a cleaning process:

- Not more than (NMT) 10 ppm detected by analytical methods such as TOC.
- NMT 1/1000 of the normal therapeutic dose.
- No visible residue.
- For certain allergens (e.g., penicillins, steroids, and cytotoxic materials), the set limit is often established below the limit of detection of the best available method.

The acceptable limit for bacterial endotoxins is generally set only for equipment used in the manufacture of parenteral and inhalation products. For rinse samples, limits are often set based on the WFI specification (i.e., <0.25 EU/mL) or based on safety requirements for the product to be manufactured. Bacterial endotoxin is difficult to measure from surfaces using swabs and, therefore, there is typically no general limit guideline for this type of test.

There are a few approaches used by companies to establish bioburden limits for equipment cleaning, all acceptable by the regulatory agencies as long as there is good scientific rationale to justify the chosen values. For rinse samples, the most common approach is to use the recommended bioburden limits for pharmaceutical-grade waters as the minimum requirement. For example, for equipment cleaned with purified water, the limit is set at NMT 100 CFU/mL, and for equipment cleaned with WFI the limit is set at NMT 10 CFU/100 mL or <1 CFU/10 mL. For surface sampling of nonsterile equipment, it is an industry practice to accept a limit in the range of 0–10 CFU/25 cm^2.

Another approach used for nonsterile product manufacturing is to apply the "next product" bioburden specification or the proposed compendial bioburden levels for nonsterile pharmaceuticals for the "next product" (see USP Chapter <1111> *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use*) to calculate the surface bioburden limit for cleaned equipment used in the manufacture of the "prior product." The following is an example calculation to illustrate this approach.

Example

Next product: Topical Formulation
Bioburden specification: NMT 100 CFU/g
Equipment surface area: 200,000 cm^2
Batch size: 100 kg
Rinse volume: 1,000 L

Calculations for CFU/cm^2 (swab):

$$\frac{100\ \text{CFU/g} \times 100\ \text{kg} \times 1000\ \text{g (conversion factor)}}{200{,}000\ \text{cm}^2}$$

Acceptable number of CFU/cm^2 of equipment = 50 CFU/cm^2

Calculations for CFU/mL (rinse):

$$\frac{\text{surface limit (swab)} \times \text{total surface area}}{\text{Rinse Volume}}$$

Where rinse volume is determined by volume used and not collected rinse volume

Calculations for CFU/mL (rinse):

$$\frac{50\ \text{CFU/cm}^2 \times 200{,}000\ \text{cm}^2}{1{,}000\ \text{L} \times 1{,}000\ \text{mL (conversion factor)}}$$

Acceptable number of CFU/mL (rinse): 10 CFU/mL

EXECUTION OF EQUIPMENT-CLEANING VALIDATION PROTOCOL

Once acceptance criteria have been established, recovery studies completed, and a cleaning validation protocol created and approved, a company is ready to execute the validation protocol for equipment cleaning. Sampling of equipment for verification of cleaning is performed after the soiled equipment (e.g., after production or soiled with product or another material for the study) has undergone the cleaning cycle. Typically, three runs are performed to show reproducibility of the cleaning methods, but results should be evaluated separately and not averaged. Some studies include sampling of equipment before and after cleaning/sanitization for an evaluation of cleaning effectiveness. During validation studies, it is also important to identify representative microorganisms isolated to establish a baseline for trending purposes and to assist during investigations into future events of bioburden recoveries from manufacturing equipment.

Results obtained from rinse and/or swab samples from all the three runs are evaluated against established limits. If the cleaning method fails to meet protocol acceptance criteria, modifications to the cleaning procedure are needed, and the cleaning validation study must be repeated.

VALIDATION OF CLEANED EQUIPMENT HOLD TIME

A cleaning validation protocol should include validation studies to establish a cleaned equipment hold time (CEHT) defined as the elapsed time from end of equipment cleaning until the time equipment is used again. Surfaces that have been cleaned and sanitized are always at risk of being recontaminated if the appropriate precautions and protocols are not followed. Therefore, this type of study will focus on the condition of the equipment at the beginning of storage (e.g., dry or wet), how, where, and for how long the equipment will be stored. In addition, the protocol must address the sampling locations based on potential routes of contamination during storage. These sampling sites may or may not be the same ones sampled during the original equipment-cleaning validation process.

A typical protocol for CEHT study includes sampling of clean equipment (rinse and/or swab) at the beginning of storage (baseline) and again at the end of the proposed storage period, using validated recovery methods. This study can be performed as part of the equipment-cleaning validation protocol or as a separate protocol, depending on the manufacturing schedule flexibility, to avoid delays in completing the cleaning validation protocol. Acceptance criteria for bioburden and bacterial endotoxin levels at the end of the storage period must meet established lim-

its for equipment cleaning. Further, for bioburden levels, most protocols require that, at the end of the storage period, the bioload does not increase by more than 0.5 log (0.3 log harmonized) from the initial bioburden (baseline).

Example Calculation (using 0.5 log variability)

Recovery for initial bioburden (or control): 63 CFU
Log_{10} 63 = 1.7993
Upper limit = antilog_{10} (1.7993 + 0.5) = 199
Lower limit = antilog_{10} (1.7993 − 0.5) = 19.9, rounds to 20
Log variability: 20 to 199 CFU

The recovery for the test sample at the end of the hold time is 57 CFU. Therefore, the test passes as 57 CFU is less than 199 CFU. Data generated from a CEHT study provide supporting documentation for the storage of cleaned equipment under the evaluated environmental conditions and for the given maximum time period.

VALIDATION OF DIRTY EQUIPMENT HOLD TIME

Another typical study performed as part of equipment-cleaning validation establishes the maximum allowed time that a piece of equipment can remain soiled prior to being cleaned. It is critical that, during this time frame, the soil remain in a physical condition that allows easy removal and no additional bioburden or bacterial endotoxin be generated above and beyond the capabilities of the cleaning and sanitization methods.

A typical protocol for dirty equipment hold time (DEHT) study includes sampling of cleaned equipment (rinse and/or swab) following the proposed storage period/dirty hold time. As a test control, sampling is also performed after the equipment is cleaned immediately following production or soiling. As with a CEHT study, the DEHT study can be performed as part of the equipment-cleaning validation protocol or as a separate protocol, depending on the manufacturing schedule flexibility, to prevent delays in completing the validation protocol. Acceptance criteria for bioburden and bacterial endotoxin levels for equipment cleaned at the end of the dirty hold period must meet established limits for cleaned equipment. Data generated from a DEHT study provide supporting documentation for the storage of soiled equipment under the given environmental conditions and for the given maximum time period prior to cleaning.

ONGOING VERIFICATION OF CLEANING

Once validation efforts are complete, and results have established the effectiveness of the cleaning and sanitization procedures, a microbiological control program for manufacturing equipment must be implemented along with a program for cleaning validation maintenance. Key considerations to ensure continued validity of the cleaning procedures include

- Need for additional validation work when new products are introduced in the plant
- Retraining of cleaning and equipment sampling personnel
- Need for revalidation whenever changes to the cleaning procedures are implemented
- Need for ongoing verification of cleaning

The cGMPs regulations require that companies perform some type of verification of cleaning prior to equipment use. In most cases, this type of cleaning verification includes visual inspection and limited chemical testing. However, depending on the type of equipment or process, additional testing including bioburden and endotoxin testing may be warranted. One such example is following area changeover that takes place between campaigns of different products. During a changeover procedure, equipment soft parts are changed and the reassembled equipment is cleaned and sanitized. Because this type of activity is conducive to introduction of bioburden into the equipment, most companies choose to collect bioburden and endotoxin (if applicable) samples after the cleaning cycle. The FDA also recommends taking bioburden rinse samples of chromatography columns prior to use to monitor for potential biofilm formation.

VALIDATION OF HOLDING TIME/SHIPPING CONDITIONS

The purpose of this study is to check that microorganisms that are viable at the time of swabbing/rinsing remain viable and stable until testing occurs in the laboratory. This study is necessary only if there is a significant lag time (e.g., more than 8 h under refrigeration) between sampling and processing of swabs or rinse aliquots.

This test is performed by inoculating the tip of the chosen type of swab, prewetted in the chosen holding/transport medium, with less than 100 CFU of one of the challenge organisms. Then, the swab is placed in a sterile tube containing the chosen sterile holding/transport medium. If rinse technique is the method of choice, inoculate 10-mL aliquots of rinse diluent contained in a sterile container with less than 100 CFU of one of the challenge organisms. Prepare three replicates for each of the challenge organisms. Include one uninoculated swab plus holding/transport medium or aliquot of rinse diluent, to serve as a negative control. Prepare triplicate test-positive controls for each test organism by inoculating replicate test tubes containing 10 mL of 0.1% peptone water or sterile saline solution with less than 100 CFU of the test inocula. Hold all sample preparations in a controlled environment that simulates the proposed holding/shipping conditions, such as the following:

- Refrigerated (2–8°C) for 8, 24, 36, or 48 h
- Room temperature (15–30°C) for 8, 24, 36, or 48 h

After the desired holding time is achieved, test the inoculated swabs and/or rinse sample preparations (as described earlier in this chapter), by the pour-plate, streak-plate, or membrane filtration method.

Perform inocula counts by plating the same inocula used in the test to verify that the initial microbial challenge was within the target range; plate selected dilutions of the inocula, in duplicate or triplicate, with TSA and incubate at 30–35°C for 2–3 d. At the end of the incubation period, enumerate the recovered microbial colonies and calculate the average number of CFU. Compare the average number of recovered colonies from the inoculated test samples with the average number of recovered colonies obtained for the test-positive controls. In addition, compare the average microbial recovery from the test-positive controls to the average inoculum plate count for the given test organism. The study acceptance criteria are as follows:

- No microbial growth is obtained for the negative control preparations.
- The average number of microorganisms recovered from the inoculated swabs or rinse samples should be within 0.5 log (0.3 log harmonized) of the average counts obtained for the test controls.
- The average number of microorganisms recovered from the test-positive control preparations should be within 0.5 log (0.3 log harmonized) of the inocula plate counts for the given test organism.

Refer to page 156 for an example calculation to determine if result is within 0.5 log variability of control. If the sample preparation and/or test control recoveries fail to meet the proposed test acceptance criteria, there is a possibility that the microbial populations are not stable and that the challenge organisms may be multiplying or losing viability under the holding/shipping conditions. In such cases, a laboratory investigation should be performed in order to improve the proposed sample hold conditions to ensure accuracy of the test data. A different combination of swab/holding medium or transport system for the rinse samples may be needed to overcome loss of microbial viability or to prevent microbial proliferation under the given sample holding/shipping conditions.

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7 Method Validation and Media Suitability Testing

The suitability of compendial microbiological methods must be demonstrated by the user to ensure accuracy and reliability of test results. This topic is addressed by the United States Pharmacopeia (USP) in the following chapters:

Chapter <1225>, Validation of Compendial Methods—According to section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the USP and the National Formulary constitute legal standards. The current good manufacturing practice (cGMP) regulations [21 CFR 211.194 (a)] require that test methods, which are used for assessing the compliance of pharmaceutical products with established specifications, must meet proper standards of accuracy and reliability.

Chapter <61>, Microbiological Examination of Non-Sterile Products: Microbial Enumeration Tests—In this chapter, it is clearly stated that a testing laboratory must establish the ability of the compendial method to detect microorganisms that might be present in the product being tested; if the product has antimicrobial properties, it is necessary to either remove or neutralize the inhibitory substance prior to performing the microbial limit test.

Chapter <62>, Microbiological Examination of Non-Sterile Products: Tests for Specified Microorganisms—In this chapter, the need is presented to ensure that the compendial method is able to detect specified organisms and that the media used in the testing contain nutritive and selective properties to detect the organisms of interest.

Chapter <1227>, Validation of Microbial Recovery from Pharmacopeial Articles—This chapter, which provides guidance for validating methods for recovery of microorganisms, emphasizes the fact that if a product possesses antimicrobial properties because of the presence of a specific preservative or due to its formulation, the antimicrobial property must be neutralized in order to recover viable microorganisms.

Requirements for validation of compendial methods are also addressed in the European Pharmacopoeia (EP) and in the Japanese Pharmacopeia (JP) within the various chapters for microbiological examination of nonsterile products. Therefore, a general understanding of the need to eliminate any antimicro-

bial property prior to evaluating a product's microbial burden is universally accepted. During a method validation study, the user demonstrates that the chosen method for the qualitative and/or quantitative estimation of viable microorganisms is sensitive, accurate, and reliable, and that it can overcome any interference and inhibition (such as prevention of spore germination and cellular division) in the recovery of viable organisms.

Since the adoption of the harmonized compendial chapters for microbiological examination of nonsterile products, users are wondering whether or not they need to revalidate their current methods. As most harmonized tests are different from previous ones, it is likely that some revalidation work will be needed. However, prior to embarking in costly and time-consuming method-validation studies, there are some considerations that companies should take in order to assess whether additional work is warranted or not. For example, changes in recovery media and incubation conditions most likely will require revalidation. However, if only changes to the minimum amount of product tested and types of challenge organisms apply, revalidation work may or may not be necessary, as long as the existing test method can be executed in a manner that meets harmonized requirements and there is scientific rationale for not doing additional work.

Companies need to be proactive and should start evaluating their existing methods against the harmonized methods in a timely manner, so that any needed revalidation work is performed and methods updated prior to the official date of implementation by the compendia. In some cases, not every registered product may require revalidation using the harmonized methods. According to the EP, companies can use a scientifically sound risk-based approach to develop a revalidation program for their approved products; where justified, a bracketing approach for a product line may also be used when selecting products to undergo revalidation work. For example, for a product having potencies equal to 3 mg/mL, 5 mg/mL, 7.5 mg/mL, and 10 mg/mL, the revalidation work can be performed using the 3 mg/mL and the 10 mg/mL products.

SUITABILITY TEST DESIGN

Method suitability studies should be performed prior to the examination of a product for its microbial content. However, some companies choose to perform the validation concurrently with the quality control test, especially if the product being tested is known not to have antimicrobial properties or if the company has prior experience with the type of product being evaluated. Concurrent validation is therefore an acceptable practice as long as the validation study is complete prior to releasing the quality control test results.

A suitability test protocol must mimic the proposed microbial limit test—the sample preparation, types of media and buffers, the number of buffer rinses as well as incubation conditions must be reproduced during validation. In order to demonstrate that the proposed method is capable of recovering viable microbes that might

be present in the product sample, the method suitability protocol requires the use of representative microorganisms to challenge the microbial recovery methods. When the test sample contains antimicrobial properties, these must be eliminated by means of dilution, washing, filtration, and/or chemical inactivation to ensure adequate recovery of viable microorganisms. It is also part of a method suitability test to demonstrate that the chosen neutralization method is not harmful or toxic to microorganisms and that the test media are suitable for the recovery of specified organisms under the given test conditions. In addition, test-negative controls are performed alongside the challenge tests to verify absence of contamination in the media and in the materials used in the study. A product-negative control is performed to evaluate any inherent product bioburden that might interfere with the recovery challenge studies.

The test design to evaluate *neutralizer efficacy* and *neutralizer toxicity* involves three treatment groups:

1. Test Group (efficacy evaluation group)—In this treatment group, the product is subjected to the test method and then inoculated with specified test organisms. Product preparation may include a neutralization step, if needed. This test will evaluate adequate recovery of representative microorganisms from the sample matrix as well as the effectiveness of the neutralization method used.
2. Peptone Control Group (toxicity evaluation group/positive control)—In this treatment group, instead of product, peptone water or another buffer solution is subjected to the same sample preparation and challenge organism inoculation used for the Test Group. If the Test Group was subjected to a neutralization step, the Peptone Control Group must also be subjected to the same neutralization method. This test is in essence a positive control that evaluates the recovery of test organisms in the absence of the product.
3. Viability Group (inoculum count verification)—In this test group, the population of the test organism inocula used to challenge Test Groups 1 and 2 is verified by the plate count method in the absence of the neutralization method and the product being tested. This test is done to ensure that the challenge test was performed using an appropriate inoculum level.

The inocula used to challenge the treatment groups must be less than 100 CFU (low-level inoculum). For microbial enumeration challenge tests, a sufficient volume of the microbial suspension must be added so that a final inoculum concentration of less than 100 CFU per inoculum volume is achieved. The inoculum should be added to the product prior to neutralization to address recovery of "injured" organisms, a topic addressed later in this chapter. However, if inhibition is encountered during suitability studies, the inoculum can be added after the neutralization step is performed. Adequate recovery of the test organisms confirms the suitability of the test method. Failure to recover the test organisms necessitates specific modifications to the test procedure.

The evaluation of the suitability of the proposed test method includes a comparison of the microbial recoveries obtained from the various treatment groups: Similar recoveries between the Test Group and Peptone Control Group indicate adequate

neutralization of microbial inhibitory properties that might be present in the sample; similar recoveries between the Peptone Control Group and Viability Group demonstrate that the neutralization method itself is not toxic to the test organisms.

Validation/suitability studies for microbial recovery methods should be performed using at least three independent sample preparations. Whenever possible, three product lots/batches should be evaluated. However, if only one product lot/batch is available, three independent studies should be carried out in order to better evaluate method variability. The method is considered validated if acceptable recovery is shown in all three replicates.

As discussed in Chapter 3 of this book, variations of the official compendial methods, including automated methods, are acceptable as long as method comparability studies demonstrate that the suggested method is equivalent or shows improved recovery of viable microorganisms. This topic will be addressed in detail later in this chapter.

REPRESENTATIVE CHALLENGE ORGANISMS

Microorganisms are available commercially from vendors such as the American Type Culture Collection (ATCC), the National Collection of Industrial, Marine, and Food Bacteria (NCIMB), the Collection de l'Institut Pasteur (CIP), the National Institute of Technology and Evaluation (NITE-BRC/NBRC), the National Collection of Pathogenic Fungi (NCPF), the Institut Pasteur (IP), and the International Mycological Institute (IMI). The following is a list of challenge organisms used for the validation studies of microbial enumeration and bioburden tests:

- *Staphylococcus aureus* (representative Gram-positive coccus) such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276
- *Pseudomonas aeruginosa (*representative nonfermenter Gram-negative rod) such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275
- *Candida albicans* (representative yeast) such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594
- *Aspergillus niger* (representative filamentous fungus) such as ATCC 16404, IMI 149007, IP 1431.83, or NBRC 9455
- *Bacillus subtilis* (representative spore-forming Gram-positive rod) such as ATCC 6633, NCIMB 8054, CIP 52.62, or NBRC 3134

The following is a list of challenge organisms used for the validation studies for screening of specified microorganisms; stock cultures are available commercially from vendors such as the ATCC, NCIMB, CIP, NITE-BRC/NBRC, NCPF, IP, and the National Collection of Type Cultures (NCTC):

- *Staphylococcus aureus,* such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276
- *Pseudomonas aeruginosa*, such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275
- *Escherichia coli*, such as ATCC 8739, NCIMB 8545, CIP 53.126, or NBRC 3972

- *Salmonella enterica* ssp. *enterica* serotype typhimurium, such as ATCC 14028; alternative *Salmonella enterica* ssp. *enterica* serotype abony, such as NBRC 100797, NCTC 6017, or CIP 80.39
- *Candida albicans*, such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594
- *Clostridium sporogenes*, such as ATCC 11437 (NBRC 14293, NCIMB 12343, or CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.03)

Environmental isolates and other types of test organisms should be included in the challenge test, especially if it appears that such microorganisms may represent contaminants likely to be introduced during manufacture or use of the product.

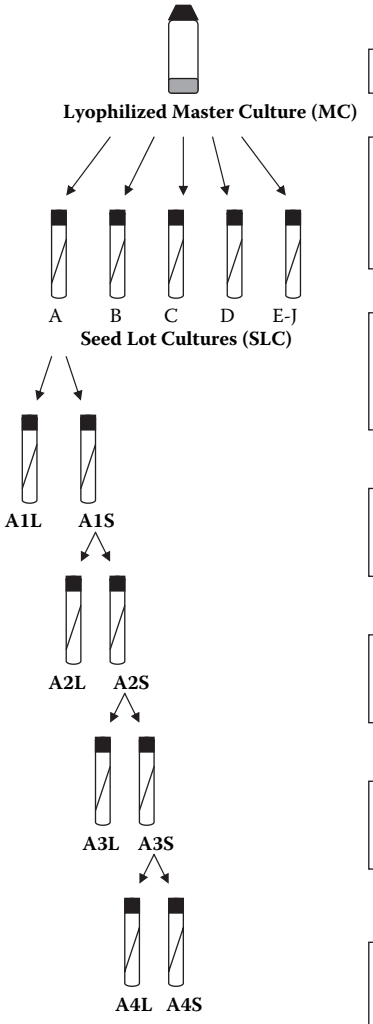
MAINTENANCE AND PREPARATION OF TEST ORGANISMS

The accuracy, reproducibility, and reliability of the method suitability tests are directly affected by the metabolic and physiological condition of the cells in the test inocula suspensions. Therefore, having a qualified and standardized protocol for stock culture maintenance and for the preparation of working test organism suspensions is a critical activity in the quality control microbiology laboratory.

Cultures of microorganisms used in compendial testing should be purchased from a national culture collection. Lyophilized cultures need to be rehydrated according to the manufacturer's directions and maintained viable through frequent transfers onto fresh media or by using a qualified freezing procedure. Alternatively, commercially available ready-to-use suspensions, such as Quanti-Cult® and Culti-Loop® (Remel, Inc., www.remel.com, United States; Oxoid Limited, www.oxoid.com/uk), as well as the BioBall® (www.bioMérieux-industry.com) are acceptable for challenge studies as long as the laboratory maintains documented evidence (vendor's certificate of analysis and quality control testing performed in-house) of the product's identity and cell population (if applicable). Refrigerated cultures are maintained by weekly or monthly transfers to fresh agar slants, plates, or stabs, which are kept under refrigerated conditions (2–8°C). Stock cultures that are maintained frozen are first suspended in a cryopreservation medium contained in vials and then stored at ≤30°C. The USP states in Chapter <1117>, *Microbiological Best Laboratory Practices*, that cultures stored at –70°C or in lyophilized form may be kept indefinitely.

Because the preparation and storage of microorganisms determine the physiological condition of the cells, microbiologists must choose the best culture maintenance method to avoid mutations and to minimize variability in microbial resistance and viability. Stock-culture-maintenance conditions are even more critical for environmental isolates, such as biofilm cells, used as challenge organisms in method validation and disinfectant qualification studies. These types of organisms quickly change their phenotypic profile once removed from the environment where they were found, leading to questionable test results.

Many laboratories use a seed-lot technique for the maintenance of stock cultures, especially for those that are stored under refrigerated conditions. Using this technique, which is illustrated in Figure 7.1, cultures are transferred at regular intervals onto fresh medium in order to maintain cell viability. Each trans-



Step 1. Reconstitute MC per manufacturer's directions

Step 2. Transfer to recommended medium (slants, stabs, or broth). Prepare (up to 10 or more) subcultures and incubate at appropriate conditions. Observe for growth; confirm identity and purity using a representative subculture. Refrigerate SLCs.

Step 3. After a given time period (e.g., one week) perform 2 subcultures from the SLC-A. Incubate subcultures at appropriate conditions. Observe for growth and discard SLC-A.

Step 4. Refrigerate A1L and A1S. Subculture A1L is made available for lab use. Subculture A1S is stored in a secure location and used for subsequent transfers.

Step 5. At regular intervals (e.g., weekly or monthly), proceed with subcultures as seen in this figure until 4 transfers are prepared.

Step 6. Once subculture of the A series is complete, start a new series of subcultures using SLC-B and so on, until all the SLC stocks have been used.

Step 7. At frequent intervals, perform quality control checks on representative subcultures to confirm cell viability, purity, identification, and expected phenotypic profile (if applicable).

FIGURE 7.1 Seed-lot technique.

fer, whether for storage or for preparation of a working culture, is considered a *passage*. The compendial recommendation is to limit the number of transfers (passages) to five in order to avoid mutations. Therefore, the author recommends limiting the number of passages during a seed-lot-technique procedure to *four*; once the fourth passage is subcultured onto fresh medium for the preparation of a working stock culture, the resulting cell suspension is considered the *fifth* passage. In addition, in order to monitor the quality of the stock cultures, the author recommends periodic quality control tests, including purity tests, phenotypic profile/biochemical reactions using selective media, and identification (if possible) to the species level.

PREPARATION OF WORKING CULTURES

Prior to each test, prepare fresh transfers using agar medium or culture broth. Grow the bacterial cultures using soybean casein digest (SCD) medium (agar or broth) incubating at 30–35°C for 18–24 h. Grow yeast cultures using sabouraud dextrose agar (SDA) medium (agar or broth) incubating at 20–25°C for 2–3 d. Grow cultures of filamentous fungi (mold) using SDA or potato dextrose agar (PDA) incubating at 20–25°C for 5–7 d or until good sporulation is achieved. Clostridia strains must be grown using reinforced medium for Clostridia (RMC) and under anaerobic conditions at 30–35°C for 24–48 h.

If transfers are prepared on solid media, harvest the bacteria and yeast cultures by washing each slant or plate with approximately 2 mL of sterile USP Saline test solution (TS), pH 7.0 buffered sodium chloride solution, or pH 7.2 phosphate buffer. If transfers are prepared in liquid media, centrifuge the suspension, decant the supernatant, and resuspend the microbial pellet in sterile USP saline TS, pH 7.0 buffered sodium chloride solution, or pH 7.2 phosphate buffer. Suspensions of bacteria may be adjusted with the buffer diluent to an optical density of 0.1–0.3 at a wavelength of 550 nm, using a spectrophotometer; yeast suspensions may be adjusted with the buffer diluent to a 5.0 McFarland turbidity standard. As a guideline, a 1-mL aliquot of the 10^{-5} or 10^{-6} dilutions of these recommended standardized suspensions of bacteria and a 1-mL aliquot of the 10^{-4} dilution of the recommended standardized suspension of yeasts will yield counts in the range of 10–100 CFU. These suggested optical densities may be varied such that the required concentration of microorganisms needed for the challenge tests is achieved.

According to the USP, if the standardized inocula of bacteria and yeasts are not used promptly (within 2 h), the suspensions must be stored under refrigeration for not more than 24 h. However, there is a guideline that suspensions of vegetative organisms prepared in USP saline TS or a buffer solution remain viable and stable for 7–10 d if maintained under refrigerated conditions. If the user chooses to follow a protocol that deviates from the one specified in the compendia, the alternate method must be validated.

Mold spores should be harvested by washing the agar surface with sterile USP saline TS or a buffer solution containing 0.05% polysorbate 80. Use a sterile inoculating loop or some sterile glass beads to loosen the spores and combine the washings in a sterile container. This is the mold inoculum. Tilt the inoculum container sideways; on viewing from underneath against a light source, the edge of the suspension should appear opaque. As a guideline, a 1-mL aliquot of the 10^{-5} or 10^{-6} dilutions of the aforementioned standardized suspension will yield mold counts in the range of 10–100 CFU. A mold spore suspension is usually stable over a period of 14 d if kept under refrigerated conditions. However, according to the compendia, validation of the proposed storage period is also required.

To prepare a bacterial spore suspension (e.g., *Bacillus subtilis*), harvest the inoculated agar plates with sterile water and heat shock the suspension for 15 min at 65–70°C, starting the timing when the temperature reaches 65°C. Cool rapidly in an ice bath (0–4°C), and store the prepared spore suspension under refrigeration. The microbiologist should perform an initial plate count to verify the spore

population. As a guideline, *Bacillus* and *Clostridium* spore suspensions remain viable and stable for about 4 weeks (if not longer) if kept under refrigerated conditions (2–8°C). However, validation of the proposed storage period is also required per the compendia.

Validation of Storage Period for Working Cultures

The validation of a proposed refrigerated storage period for working microbial suspensions (used in challenge studies) can be achieved by performing several plate counts of the stored inocula over time. Perform the first plate count on the day the suspension is prepared (initial count), then at regular time intervals, and again at the final proposed time point. A 0.5-log (0.3 log harmonized) variability in counts between test time points can be used as a measure to confirm *no changes in count*. A drop in the microbial population by more than 0.5 log (0.3 log harmonized) from initial count should be an indication that the given time point has exceeded an appropriate storage period for the given organism suspension. Duplicate or triplicate testing is recommended to account for normal plate count variability so that a better assessment of an appropriate storage period can be made. If applicable, the validation protocol should include verification of expected biochemical reactions on selective media.

The length of a storage period for microbial suspensions is highly dependent on the type of microorganism. Even when storage conditions are validated, unexpected changes in microbial viability may occur. Therefore, prior to performing a challenge test, the microbiologist should monitor the viability of the inocula suspensions (recommended within 24 h of testing) for some guidance on which dilution of the organism stock suspension should be used to inoculate the test samples. For the purpose of checking microbial viability, serial dilutions of the microorganism suspensions are prepared using sterile USP Saline TS or a buffer solution. Aliquots from each dilution are then plated using an appropriate agar medium and incubated at suitable conditions. Whether choosing to perform this recommended pretest viability check or not, the population of the inocula suspensions *must* be verified on the day of the test (viability group).

Recovery of Injured Organisms

Laboratory cultures used in method suitability studies are considered "healthy" organisms because they have never been exposed to antimicrobial agents or other stress conditions. Therefore, the recovery of injured (stressed) organisms that may be present in a product must be addressed when designing a method validation protocol. The compendia recommend challenging the product prior to the neutralization step as this procedure represents the greatest stress to the healthy lab cultures. If, on using this inoculation approach, inhibition of growth cannot be avoided, the compendia find it acceptable to add the microbial challenge after neutralization (chemical reaction, dilution, or filtration). However, even when inoculation is done post neutralization, the microbiologist can still perform a modified stress challenge by holding back the inoculated sample preparations for a given period of time, not to exceed one hour, prior to processing. This approach is recommended by the author

because it demonstrates that viable microorganisms able to survive in the product matrix can be recovered, and it also serves to qualify the sample preparation hold time for routine testing.

Recovery of injured organisms is addressed in the USP Chapter <1227> in which it is stated that if a company chooses to use an alternate recovery medium, a comparison study to verify the recovery of injured organisms from a sample matrix using the alternate and compendial (preferred) medium should be performed. If recovered counts from the alternate medium are within 0.5 log (0.3 log harmonized) units when compared to the preferred medium, the proposed alternate method is considered acceptable and validated.

SUITABILITY TESTING BY DIRECT INOCULATION/PLATING METHODS

Traditional microbiological testing involves detection of microorganisms via direct inoculation into liquid media (enrichment broths) and direct plating using agar media. Direct inoculation into enrichment broths are typically performed to test for the absence or presence of specified microbial species. Direct plating for the purpose of microbial enumeration can be performed either by pour-plate or spread-plate techniques.

Validation of Screening for Specified Microorganisms

To prepare the *Test Group*, dilute the product as directed in the proposed microbial limit test, typically starting at a 1:10 product dilution. Refer to Tables 7.1, 7.2, 7.3, and 7.4 for example sample preparation schemes. The enrichment broth used (e.g., tryptic soy broth [TSB]) may contain chemical inactivators if the product being tested contains antimicrobial properties. Typically, 0.5% lecithin and 4% polysorbate 20 are used as general neutralizers for enrichment and recovery media. See Table 7.5 for a list of other types of neutralizing agents that can be added to test diluents and recovery media. For some interfering substances, such as alcohols, dilution can be used as a means to reduce or remove antimicrobial properties in the sample.

After the product is diluted, shake or mix the sample preparations well to create a homogeneous solution or suspension. Separately inoculate each sample preparation with a low-level inoculum (less than 100 CFU) of a pure culture of each specified test organism. The inoculum-to-test ratio should not exceed 1% (e.g., 1 mL of inoculum per 100 mL of sample preparation). As addressed earlier, inoculation is done prior to or after the neutralization step (after the product is diluted and/or added to the enrichment medium containing neutralizing chemicals).

Prepare the Peptone Control Groups (test-positive controls) by using the same volumes, types of media, and inoculum levels used for the preparation of the Test Groups (product preparation) but replacing the sample with a buffer diluent such as peptone water. Prepare the Viability Group by plating the same volume of organism suspension used to inoculate the Test Groups and Peptone Control Groups by the pour-plate or spread-plate method. In general, bacterial suspensions are plated with SCD agar and incubated at 30–35°C for 24–48 h. For fungi, use SDA medium

TABLE 7.1
Example of sample preparations for the method suitability testing for absense of *S. aureus, P. aeruginosa, E. coli,* and *C. albicans*.

Table 7.1A. Procedure Option 1:

- Test the equivalent for one (1) gram or one (1) ml of product by adding 10 mL aliquots of the product in buffer preparation to TSB or SDB. For example, prepare a **1:10 dilution** (e.g., 10 grams or 10 mL of product into 90 mL of buffer).
- Inoculate each preparation with < 100 CFU prior to or after addition of product to enrichment broth.
- Incubate and proceed as directed in the compendial method.

	Product Dilution in Enrichment Broth				
	1:10	**1:20***	**1:30***	**1:50***	**1:100***
Product in buffer volume (mL)	10	10	10	10	10
Enrichment Broth volume (mL)					
For Bacteria: use TSB (with or without neutralizers)	100	200	300	500	1000
For Yeast: use SDB (with or without neutralizers)					

* Optional if the 1:10 sample preparation is inhibitory.

Table 7.1B. Procedure Option 2:

- Prepare a 1:10 dilution (e.g., 10 grams or 10 mL of product into 90 mL of buffer).
- Reduce product amount *for validation work only*, maintaining the ratio of product amount to volume of TSB or SDB.
- Inoculate each preparation with < 100 CFU *after* addition to broth medium (neutralization step). This will mimic the product to broth ratio in the actual test.
- Incubate and proceed as directed in the compendial method.

Note: For routine testing, a minimum of one (1) gram or one (1) mL of product must be sampled, using a larger volume of broth to maintain the validated ratio.

	Product Dilution in Enrichment Broth				
	1:10	**1:20[a]**	**1:30[a]**	**1:50[a]**	**1:100***
Product in buffer volume (mL)	1	1	1	1	1
Enrichment Broth volume (mL)					
For Bacteria: use **TSB** (with or without neutralizers)	10	20	30	50	100
For Yeast: use **SDB** (with or without neutralizers)					

* Optional if the 1:10 sample preparation is inhibitory.

TABLE 7.1 (Continued)
Example of sample preparations for the method suitability testing for absense of *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans*.

Table 7.1C. Procedure Option 3:

- Reduce product amount *for validation work only*, maintaining the ratio of product amount to volume of TSB or SDB.
- Inoculate each preparation with < 100 CFU *after* addition to broth medium (neutralization step). This will mimic the product to broth ratio in the actual test.
- Incubate and proceed as directed in the compendial method.

Note: For routine testing, a minimum of one (1) gram or one (1) mL of product must be sampled, using a larger volume of broth to maintain the validated ratio (minimum of 1 g or 1 mL of product into 100mL TSB or SDB).

	Product Dilution in Enrichment Broth				
	1:10	**1:20***	**1:30***	**1:50***	**1:100***
Product amount (g or mL)	0.1	0.1	0.1	0.1	0.1
Enrichment Broth volume (mL) **For Bacteria:** use TSB (with or without neutralizers) **For Yeast:** use **SDB** (with or without neutralizers)	10	20	30	50	100

* Optional if the 1:10 sample preparation is inhibitory.

TABLE 7.2
Example of sample preparations for the method suitability testing for absence of *Salmonella* spp.

Table 7.2A. Procedure Option 1:

- Test the equivalent of 10 grams or 10ml of product in TSB.
- Inoculate each preparation with < 100 CFU prior to or after addition to broth medium (neutralization step).
- Incubate and proceed as directed in the compendial method.

	Product Added Directly to TSB				
	1:10	**1:20***	**1:30***	**1:50***	**1:100***
Product amount (g or mL)	10	10	10	10	10
TSB volume (mL) (with or without neutralizers)	100	200	300	500	1000

* Optional if the 1:10 sample preparation is inhibitory.

Continued

TABLE 7.2 (Continued)
Example of sample preparations for the method suitability testing for absence of *Salmonella* spp.

Table 7.2B. Procedure Option 2:

- Reduce product amount *for validation work only*, maintaining the ratio of product amount to volume of TSB.
- Inoculate each preparation with < 100 CFU *after* addition to broth medium (neutralization step). This will mimic the product to broth ratio in the actual test.
- Incubate and proceed as directed in the compendial method.

Note: For routine testing, a minimum of 10 grams or 10 mL of product must be sampled and using a larger volume of TSB to maintain the validated ratio.

	Product Added Directly to TSB				
	1:10	**1:20***	**1:40***	**1:50***	**1:100***
Product amount (g or mL)	1	0.5	0.25	0.2	0.1
TSB volume (mL) (with or without neutralizers)	10	10	10	10	10

* Optional if the 1:10 sample preparation is inhibitory.

TABLE 7.3
Example of Sample Preparation for the Method Suitability Testing for Absence of Clostridia

Procedure:

- Prepare a 1:10 dilution of product in Buffer (e.g., 10 grams or 10mL of product into 90 mL of buffer).
- Take two aliquots equivalent to one (1) gram or one (1) mL of product.
- Inoculate each preparation with < 100 CFU (prior to heat shock and at room temperature).
- Heat shock one of the two sample aliquots/preparations.
- Add the sample aliquots (heated and not heated) to separate containers with Reinforced Medium for Clostridia (RMC).
- Incubate and proceed as directed in the compendial method.

	Product Dilution in RMC		
	1:10	**1:20[a]**	**1:30***
Product in buffer volume (mL)	10 (heated) 10 (not heated)	10 (heated) 10 (not heated)	10 (heated) 10 (not heated)
RMC volume (mL)	100	200	300

* Optional if the 1:10 sample preparation is inhibitory.

TABLE 7.4
Example of sample preparations for the method suitability testing for absence of bile-tolerant gram-negative bacteria

Table 7.4A. Procedure Option 1:

- Prepare 1:10 product dilution replicates (A, B, C, D, E) in TSB (1 gram or 1mL of product into 10 mL of TSB.
- Inoculate each preparation with < 100 CFU and incubate at 20-25°C for 2-5 hours (microbial resuscitation step).
- Add pre-incubated sample preparation to Enterobacteria Enrichment Broth-Mossel (MEEB).
- Incubate and proceed as directed in the compendial method.

	Product Dilution in MEEB				
	1:10	**1:20***	**1:30***	**1:50***	**1:100***
Product in TSB volume (mL) (TSB with or without inactivators)	A 10	B 10	C 10	D 10	E 10
MEEB volume (mL)	100	200	300	500	1000

* Optional if the 1:10 sample preparation is inhibitory.

Table 7.4B. Procedure Option 2:

- Prepare 1:10 product dilution: 10 grams or 10mL of product into 90mL of TSB.
- Inoculate the preparation with < 100 CFU and incubate at 20-25°C for 2-5 hours (microbial resuscitation step).
- Add 10-mL aliquots (equivalent to one gram or 1mL of product) of the pre-incubated sample into MEEB.
- Incubate and proceed as directed in the compendial method.

	Product Dilution in MEEB				
	1:10	**1:20***	**1:30***	**1:50***	**1:100***
Product in TSB volume (mL) (TSB with or without inactivators)	10	10	10	10	10
MEEB volume (mL)	100	200	300	500	1000

* Optional if the 1:10 sample preparation is inhibitory.

Continued

TABLE 7.4 (Continued)
Example of sample preparations for the method suitability testing for absence of bile-tolerant gram-negative bacteria

Table 7.4C. Procedure Option 3:

- Prepare 1:10 product dilution: 10 grams or 10mL of product into 90mL of TSB.
- Inoculate the preparation with < 100 CFU and incubate at 20-25°C for 2-5 hours (microbial resuscitation step).
- Add the TSB product preparation to MEEB. Reduce product amount *for validation work only*, maintaining the ratio of product amount to volume of MEEB.
- Incubate and proceed as directed in the compendial method.

Note: For routine testing, a minimum of one (1) gram or one (1) mL of product must be sampled using a larger volume of MEEB to maintain the validated ratio.

	Product Dilution in MEEB				
	1:10	**1:20[a]**	**1:30[a]**	**1:50[a]**	**1:100[a]**
Product in TSB volume (mL) (TSB with or without inactivators)	1	1	1	1	1
MEEB volume (mL)	10	20	30	50	100

* Optional if the 1:10 sample preparation is inhibitory.

and incubate the plates at 20–25°C for 3–5 d. The author recommends reading mold plates after 3 days of incubation to prevent inaccurate counts due to plate overgrowth. This test is performed to confirm the low-level challenge required for the test.

Prepare a *test-negative control* by setting aside an unopened container of the same lot of enrichment medium (or an aliquot of the medium) used in the test. Incubate the Test Groups, Peptone Control Groups, and Negative Control in accordance with the proposed test methods and proceed as directed in the compendial test for specified microorganisms. At the end of the incubation period, observe broths for turbidity and selective media for characteristic growth. Compare the microbial recovery obtained from the Test Groups and the Peptone Control Groups. See Figure 7.2 for an example challenge test for specified microbial species. The test is valid if

- The challenge organism is recovered from the Peptone Control Groups
- The Viability Groups confirm a low-level challenge (less than 100 CFU)
- The test-negative control shows absence of microbial contamination

The proposed test method is suitable for the product being evaluated if microbial growth recovered from the Test Group is comparable to the microbial growth obtained from the Peptone Control Group. Microbial growth is compared in appearance and biochemical reactions using selective media (if applicable).

If multiple product dilutions are attempted concurrently, tabulate the test results and choose the lowest product dilution that yielded a microbial recovery comparable

TABLE 7.5
Common Neutralizing Agents

Inhibitory Substance	Neutralizing Agent
Alcohols	Dilution
Aldehydes	Dilution, thiosulfates, glycine
Benzalkonium chloride	Letheen (broth or agar)
Beta lactam antibiotic	Beta-lactamase
Bis-biguanides	Lecithin
Chloramphenicol	Chloramphenicol acetyltransferase
Chlorhexidine	Sodium oleate
EDTA and related chelating agents	Dilution, Mg^{+2}, Ca^{+2}
Glutaraldehydes	Glycine, sodium hydrogensulfite
Iodine and chlorine	Sodium thiosulfate
Mercurials	Thioglycollate, sodium hydrogensulfite
Organic acids and their esters	Dilution and polysorbates
Parabens	Letheen (broth or agar), lecithin, polysorbate
Phenolics	Dilution and polysorbate
Quaternary ammonium compounds (QACs)	Lecithin, polysorbate, and letheen (broth or agar)
Sorbate	Dilution
Sulphonamides	*p*-aminobenzoic acid
Trimethoprim	Thymidine

Note: Thioglycollate can be toxic to certain microorganisms, especially spores and staphylococci; thiosulfate can be toxic to staphylococci. Dey-Engley (DE) is often used as a general purpose neutralizing medium.

to the test-positive control (Peptone Control Group). This is the dilution that should be used for routine product testing to screen for the given microbial species. In case the product specification requires screening for more than one microbial species and the enrichment medium used is the same, select the lowest product (i.e., highest product concentration) dilution at which satisfactory recovery was obtained for all test organisms. In the example given in Table 7.6, a 1:20 product dilution should be chosen for routine testing. Using this example, the same sample preparation can be incubated in TSB and streaked onto cetrimide and mannitol salt agars for detection of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively.

Using another example, if a product requires testing for absence of *Salmonella* spp. in addition to a test for absence of *Escherichia coli* and the company chooses to test for both microbial species using the same sample preparation, the challenge study with *E. coli* must be performed in a manner so that testing of a minimum of 10 g or 10 mL of product (requirement for *Salmonella* spp. screening) is done. As one can see, the method suitability test design can be customized to reflect the product specifications, the amount of product available for testing, and the routine test method chosen by the company. The examples given in this chapter may serve as guidelines when preparing method validation protocols.

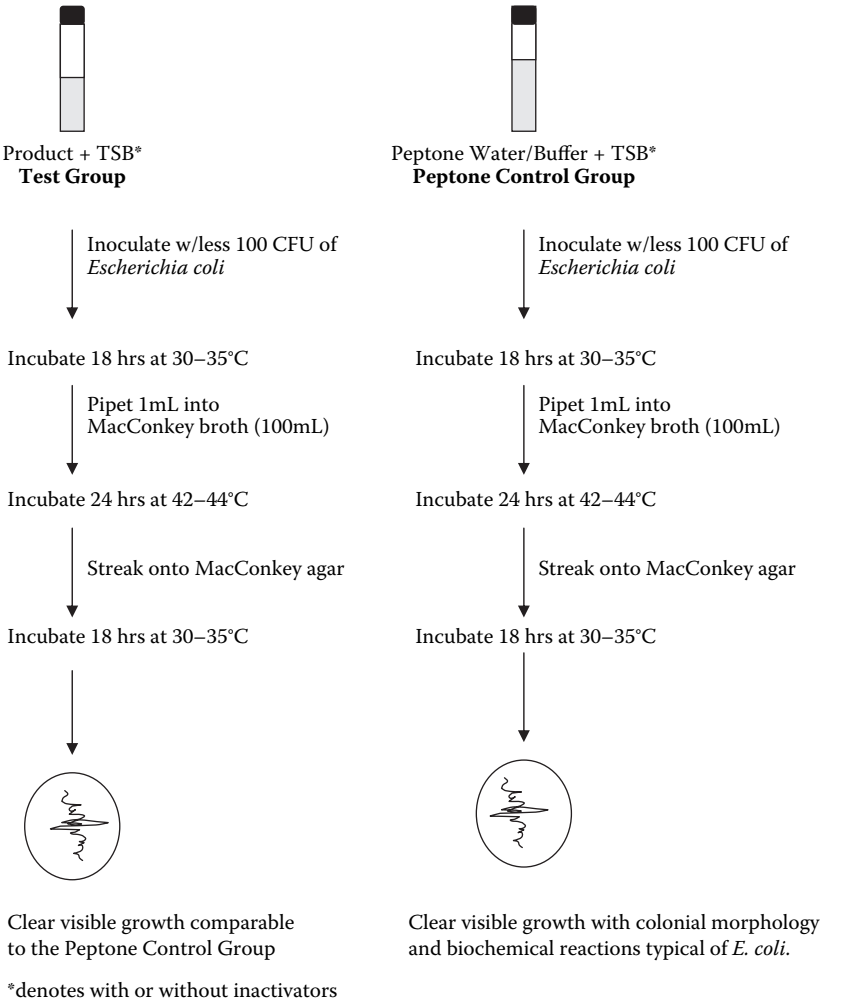


FIGURE 7.2 Suitability testing for absence of *Escherichia coli* by direct inoculation.

Modifications to the Direct Inoculation Method

Failure to recover the test organisms or meet the test acceptance criteria under the test conditions necessitates some of the following modifications:

- Add inoculum after neutralization step (chemical reaction, dilution, or filtration).
- Increase the volume of diluent.

TABLE 7.6
Example Method Suitability Test Results for Absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* for a Topical Cream

		Recovery			
		Sample Dilution in TSB			Positive Control
Test Organism	Inoculum Level (CFU)	1:10	1:20	1:40	1:10, 1:20, 1:40
S. aureus	52	+	+	+	+
P. aeruginosa	45	–	+	+	+

Note: "+" denotes growth comparable to positive control. "–" denotes no growth.

- Incorporate a sufficient quantity of a suitable inactivator in the diluent and recovery media.
- For soluble products, attempt an adaptation of the membrane filtration method.

If, following further attempts to recover a given challenge organism, the antimicrobial activity of the sample cannot be neutralized and microorganism cannot be recovered, then it is assumed that the product is likely not to be contaminated with the given microbial species. The additional data generated also provide assurance that the method suitability study was carried out using sound scientific principles and, therefore, the method is considered adequately challenged and validated. It is up to the manufacturing company to decide whether or not to perform routine testing to screen for the specified microorganisms as a precautionary measure; in some cases, small changes in product formulation may occur and those may impact survivability of microorganisms in the product matrix. In addition, routine testing using the method that produced the best recovery for at least some of the challenge organisms or using the highest product dilution attempted may permit isolation of resistant strains that, otherwise, would not be detected.

Validation of the TAMC and TYMC Tests

Approach 1

In case the product being tested requires bioburden determination as well as screening for indicator organisms, the test diluent used for the total aerobic microbial count (TAMC) and total combined yeasts and molds count (TYMC) tests can be the same as the enrichment medium used for the microbial screening test. Using this approach, for some method requirements, a smaller quantity of product would be required for routine testing as all tests can be performed using only one initial sample preparation. In addition, the data obtained during method suitability work for

microbial screening tests can be used as a starting point for the method suitability work for TAMC and TYMC. Thus, only one dilution is necessary for the validation of TAMC and TYMC tests, instead of multiple attempts.

In general, the lowest product dilution (i.e., highest product concentration) at which the test organisms are recovered in SCD broth (same as TSB) will be an acceptable dilution for the enumeration tests. See Figure 7.3 for an example testing scheme for the microbiological examination of Lactose Monohydrate, USP. Since testing for *Salmonella* spp. requires sampling a minimum of 10 g of material, screening for *E. coli* will also be performed using a minimum of 10 g of lactose. Therefore, when performing the validation work, the microbiologist would have to design the study in a manner to reflect testing of 11 g of material for the testing of both *E. coli* and *Salmonella* spp. as well as TAMC and TYMC.

Approach 2

If the product only requires microbial enumeration, a suitable buffer can be used as the test diluent. Using this approach, various product dilutions may need to be prepared during the suitability testing for TAMC and TYMC in order to determine the noninhibitory product dilution. On the positive side, this approach may also offer an advantage in terms of product amount used for routine testing; for example, because the first step in most of the harmonized screening tests for specified microbial species involves diluting the product in a buffer solution, this approach may be used when testing concurrently for microbial enumeration and absence of specified organisms. See Figure 7.4 for an example testing scheme for the microbiological examination of Corn Starch, USP.

Whether using a buffer or enrichment medium as the initial test diluent, the protocol for suitability testing is the same. To prepare the Test Groups, dilute, dissolve, or homogenize the product in the chosen diluent. Start at a 1:10 product dilution or use a dilution previously shown to be able to overcome any inhibitory properties present in the sample. Then, shake or mix the sample preparations well to create a homogeneous solution or suspension. If necessary, adjust the pH in the range 6–8. Dispense equal volume aliquots of the sample prepared into separate sterile test tubes. Prepare the Peptone Control Groups (test-positive controls) by using the same volumes of test diluent but replacing the sample with a buffer solution, such as peptone water.

For the TAMC challenge test, inoculate each test dilution separately with pure cultures of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Aspergillus niger*, and *Candida albicans*. For the TYMC test, separately challenge each test dilution with pure cultures of *Aspergillus niger* and *Candida albicans*.

As discussed earlier, the inoculum may be added to the product prior to or after the dilution/neutralization step, and the inoculum-to-test ratio should not exceed 1% (e.g., 0.1 mL of inoculum per 10 mL of sample preparation). The accepted range for countable colonies on a standard agar plate for bacteria and yeast is 25–250 CFU and 8–80 CFU for molds. This range was established by the food industry for counting coliforms in milk and proposed by the USP in the general informational Chapter <1227>. Crowding decreases accuracy, and as the number of CFU decreases, ran-

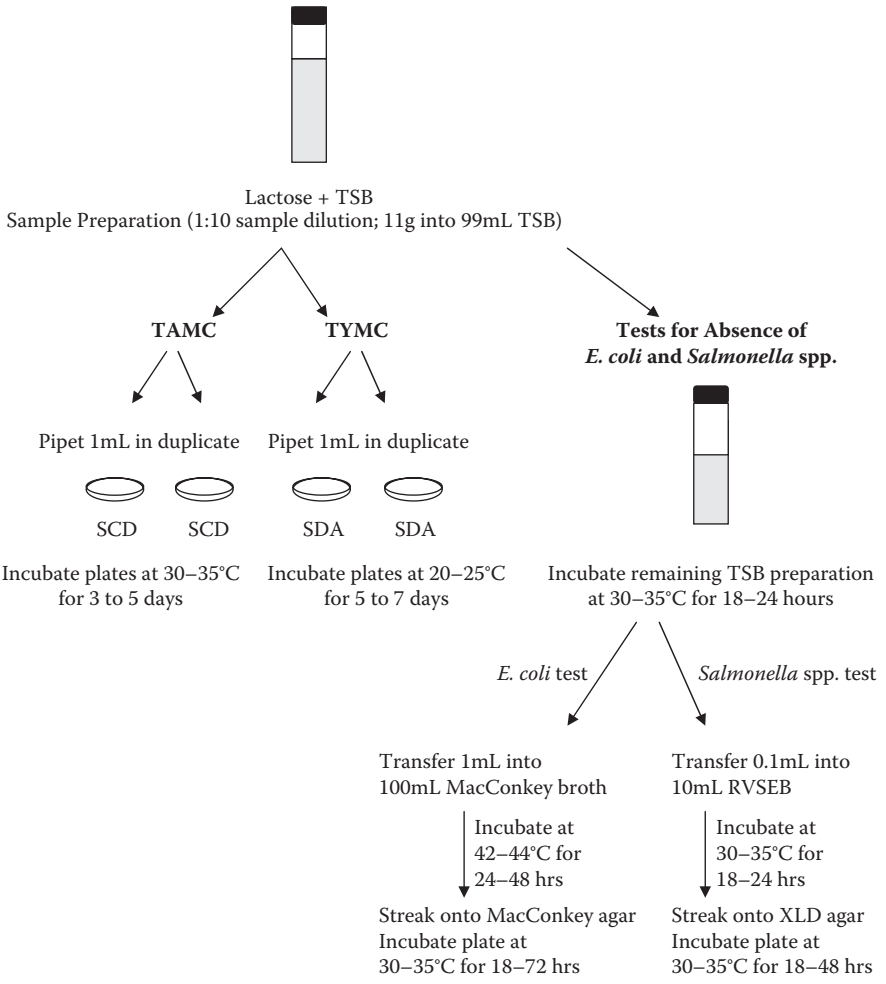


FIGURE 7.3 Example routine microbial limit testing of Lactose Monohydrate, USP.

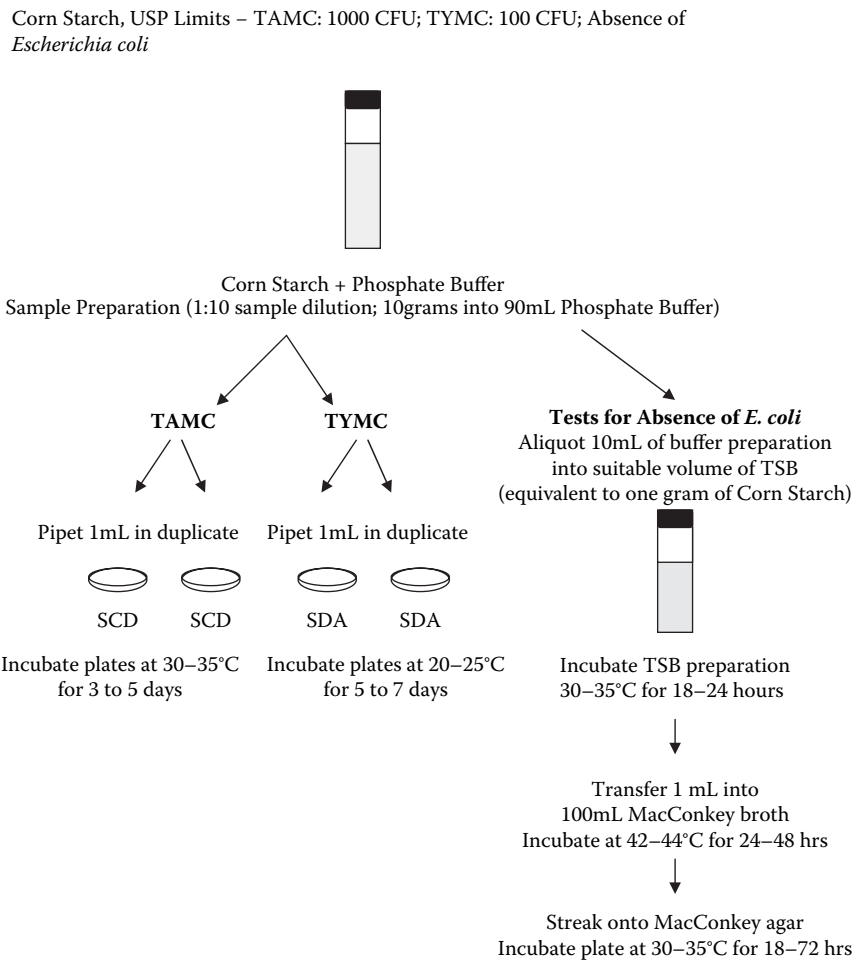


FIGURE 7.4 Example routine microbial limit testing of Corn Starch, USP.

dom error increases (see Table 7.7; adapted from the USP; Chapter <1227>, Table 2). Therefore, make sure an appropriate inoculum is used so that the expected plate count will not be too low and will not exceed 250 CFU for bacteria and yeast, or 80 CFU for molds. For example, for a 1:20 product dilution (e.g., 1 mL of product into 19 mL diluent) where 2 mL sample aliquots will be plated, challenge the sample preparation with a bacterial inoculum in the range of 250–2000 CFU. This will ensure that recovered counts will be in the range of 25–250 CFU, and that the challenge does not exceed 100 CFU/mL of sample preparation (compendial requirement).

After adding the inocula to the Test Groups and Peptone Control Groups, hold the inoculated preparations prior to plating for a time not to exceed 1 h (the author recommends holding the samples for about 15–20 min). During this holding period, the test organisms will be exposed to the product and the inactivating system. As explained earlier, this procedure will serve to address recovery of injured organ-

TABLE 7.7
Error as Percent of Mean for Plate Counts

CFU/Plate	Error as % of Mean	CFU/Plate	Error as % of Mean
30	18.3	15	25.8
29	18.6	14	26.7
28	18.9	13	27.7
27	19.2	12	28.9
26	19.6	11	30.2
25	20.0	10	31.6
24	20.4	9	33.3
23	20.9	8	35.4
22	21.3	7	37.8
21	21.8	6	40.8
20	22.4	5	44.7
19	22.9	4	50.0
18	23.6	3	57.7
17	24.3	2	70.7
16	25.0	1	100.0

Source: Adapted from USP Chapter <1227>, Validation of Microbial Recovery from Pharmacopeial Articles.

isms and will qualify the maximum elapsed time between sample preparation and plating with nutrient media. Following the proposed holding period, plate aliquots from the Test Group and Peptone Group preparations (in duplicate or triplicate) with appropriate media for the test under evaluation; use SCD medium, with or without inactivators, for the TAMC test; use SDA, with or without inactivators, for the TYMC test. See Table 7.8 for suggested aliquots to be plated for various example sample dilutions.

Prepare a test-negative control by setting aside an unopened container of the same lot of diluent (or an aliquot of the diluent) used in the test. Plate the same volume used to test the sample preparation and use the same lot of recovery medium. It is also recommended to prepare a *product negative control* to evaluate any inherent product bioburden that could interfere with enumeration and evaluation of the recovered challenge organisms. A product negative control is prepared as described for the Test Group, but without the addition of test organisms.

Prepare a Viability Group by plating the inoculum as used (same dilution scheme) to challenge the Test Groups and Peptone Control Groups using a suitable buffer as diluent (with or without inactivators) and SCD agar or SDA (without inactivators) as the plating medium. Incubate the TAMC plates at 30–35°C for 3 d (USP states to incubate bacteria for not more than [NMT] 3 d and fungi for NMT 5 d), and the TYMC plates at 20–25°C for NMT 5 d. See Figure 7.5 for an example challenge test using a plating method.

TABLE 7.8
Example Plating Schemes for TAMC and TYMC Testing

Product Dilution	Volume Plated in Duplicate (mL)	Result Calculation (Average or Total)	Test Sensitivity (No Microbial Growth Detected)
1:10	1	average	<10 CFU/ g or mL
1:10	0.1	average	<100 CFU/g or mL
1:10	0.5	total[a]	<10 CFU/g or mL
1:20	2	average	<10 CFU/g or mL
1:40	2	average	<20 CFU/ g or mL
1:40	4	average	<10 CFU/g or mL
1:100	5	total[a]	<10 CFU/g or mL
1:100	1	average	<100 CFU/g or mL

Note: If plating volumes are equal to or greater than 4 mL (pour-plate method) and 1 mL (spread-plate method), use larger Petri dishes (150 × 15 mm). Care must be taken to prevent dilution of agar due to large sample aliquot.

[a] Calculating the total of number of CFU from replicate plates is not recommended by the compendia, and should be used and justified only as a last resort. The mean value is used to account for normal plating variability in plate counts.

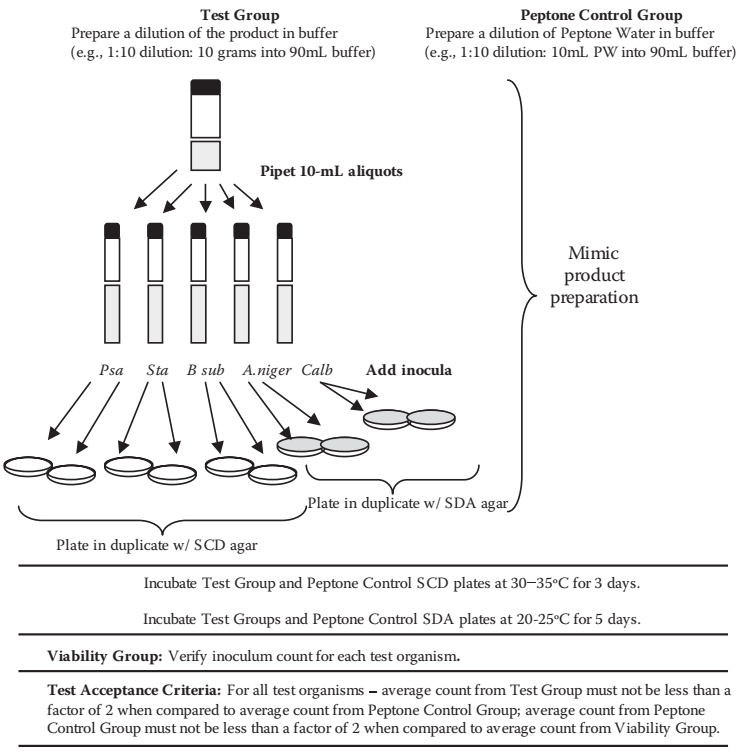


FIGURE 7.5 Suitability testing for TAMC and TYMC by pour-plate method.

TABLE 7.9
Example Microbial Recovery Calculations

	Test Group			Peptone Control Group			Neutralizer Effectiveness Evaluation
	Plate 1 (CFU)	Plate 2 (CFU)	Mean (CFU)	Plate 1 (CFU)	Plate 2 (CFU)	Mean (CFU)	Pass/Fail
Example 1	35	38	37	42	47	45 Reduction by factor of 2 = 23	PASS (37 > 23)
Example 2	35	38	37	78	82	80 Reduction by factor of 2 = 40	FAIL (37 < 40)

	Viability Group				Neutralizer Effectiveness Evaluation
	Plate 1 (CFU)	Plate 2 (CFU)	Mean (CFU)	Points to Consider	Pass/Fail
Example 1	95	99	97 Reduction by factor of 2 = 49	• In these two examples, both methods do not meet method suitability testing requirements and additional work is required. • For Example 1, the neutralizing system needs to be changed as it shows microbial toxicity. • For Example 2, the neutralizing system is not toxic but it is not sufficient to overcome the product's antimicrobial properties.	FAIL (45 < 49)
Example 2	85	87	86 Reduction by factor of 2 = 43		PASS (80 > 43)

Notice that the maximum incubation period for the validation studies is actually the minimum incubation period for routine testing. This is because standardized laboratory cultures should be readily recovered within the minimum incubation period if test conditions are suitable for microbial recovery.

At the end of incubation, enumerate the recovered colonies from the Test Group, Peptone Control Group, and Viability Group plates. Calculate the arithmetic mean of the number of colonies recovered on the replicate plates, and report the results in whole CFU numbers. For each test organism, evaluate the microbial recoveries as follows: if the mean count for the Test Group is not less than a factor of 2 when compared to the mean count for the Peptone Control Group, the method shows adequate neutralizer effectiveness; if the mean count for the Peptone Control Group is not less than a factor of 2 when compared to the mean count for the Viability Group, the method shows lack of neutralizer toxicity. See Table 7.9 for an example data evaluation.

The test results obtained for the negative control samples (buffer/media and product) must also be evaluated. For a valid test, the plates for the test-negative con-

trol (buffer/media) should be free from microbial contamination. The plates for the product negative control should be free from microbial contamination or have a very low bioburden that will not interfere with the challenge studies (inherent bioburden is easily discernable from challenge microbial colonies).

Modifications to the Plate Method

Failure to recover the test organisms or meet the test acceptance criteria under the test conditions necessitates some of the following modifications:

- Increase in the volume of diluent.
- Incorporation of sufficient quantity of suitable inactivators in the diluent and recovery media.
- Reducing the sample hold time for the inoculated sample preparations.
- Performing the microbial challenge directly into Petri dishes (no holding time). This is accomplished by adding the inocula to the Petri plate after an aliquot of the product dilution is added to the dish, mixing the preparation and adding the recovery medium shortly after.
- For soluble products, attempting an adaptation of the membrane filtration method.

If following further testing attempts no suitable neutralization method is found so that challenge organisms can be adequately recovered, it can be assumed that the inherent microbiocidal activity of the product will likely prevent contamination by the given microbial species. The additional data generated also provides assurance that the method suitability study was carried out using sound scientific principles and, therefore, the method is considered adequately challenged and validated. However, because it is possible that the spectrum of microbial inhibition is limited to the chosen test organisms, the manufacturing company should consider performing routine testing for bioburden using the highest product dilution compatible to microbial growth and one that is capable of demonstrating compliance with product specifications. For example, if a product specification is <10 CFU/g and the company chooses to perform the test using a 1:100 product dilution by plating 1 mL of sample preparation, in duplicate, and reporting the average count, the test sensitivity will be <100 CFU/g when no counts are detected (dilution factor is 100). Therefore, this testing scheme is not suitable to demonstrate compliance with the product specification of <10 CFU/g.

SUITABILITY TESTING FOR MEMBRANE FILTRATION METHODS

The compendial membrane filtration method is a modification of the sterility test. This technique uses membrane filters having an average diameter of 47 mm, a nominal porosity not greater than 0.45 μm, and microbial retentive properties that have been established and demonstrated by the vendor. Cellulose nitrate filters are typically used for aqueous, oily, and weakly alcoholic solutions; cellulose acetate filters are typically used for strongly alcoholic solutions. Special filters may be needed

depending on the product formulation. Whenever in doubt, consult the filter manufacturer. Using the membrane filtration method, the product is filtered and the membrane filter is rinsed with at least 3 × 100 mL of a benign diluent. Then, the filter is placed into a nutrient medium or onto an agar medium for recovery of any viable cells trapped on the filter membrane. Chemical neutralizers can be added to the product diluent and/or rinse fluid to enhance the recovery of the challenge organisms. The membrane filtration method offers the advantage of minimal or no product interference. In fact, filtration is listed as a suitable means to remove antimicrobial agents from sample preparations. This is because during filtration and when using the correct type of filter for the product being tested, the sample is filtered along with any chemical inhibitors, and all that is left on the membrane filter are microbial cells that might be present in the product.

For the challenge tests, the inoculum is added to the third rinse (or final rinse) aliquot and filtered. Alternatively, the inoculum may be added directly to the product prior to dilution or filtration. However, as explained earlier, adding the inoculum directly to the product represents an added stress to the challenge organisms and recovery may not be satisfactory. Therefore, the author recommends adding the low-level inoculum to the last 100 mL rinse aliquot after the product is filtered. When performing a challenge test by membrane filtration, there is no need to adjust the inoculum based on sample dilution as the entire sample preparation is filtered. However, the author recommends a target inoculum in the rage of 25–100 CFU to avoid greater than normal plating variability in test results and crowding of filter membranes.

Validation of Screening for Specified Organisms

During the suitability testing for specified organisms, only one membrane filter is required per test organism and for each Test Group. To prepare the Test Groups, dissolve/dilute 10 g or 10 mL of product into 90 mL of a suitable buffer diluent (1:10 sample dilution). Filter a 10-mL aliquot (equivalent to 1 g or mL of product) through a 0.45 µm membrane filter. *Note:* If screening for *Salmonella* spp., ensure that a minimum of 10 g or 10 mL of product is sampled. Then, rinse the membrane filter with at least two 100-mL portions of a chosen buffer, with or without inactivators. Add the inoculum to the final rinse aliquot and filter the preparation.

The Peptone Control Group is prepared by adding 10 mL of peptone water or buffer into 90 mL of the same buffer used to dissolve/dilute the product. A 10-mL aliquot of this buffer preparation is then filtered through a 0.45-µm membrane filter, and the membrane filter is rinsed with at least two 100-mL portions of the chosen buffer, with or without inactivators. The low-level inoculum (less than 100 CFU) is then added to the final 100-mL rinse and filtered.

After filtering the inoculated test preparations, each membrane filter is removed and placed into separate containers with the enrichment medium specified in the compendial method. For example, for the test for absence of *Staphylococcus aureus*, the membrane filter is placed into 100 mL of SCD broth and incubated as described in the compendia, *using the shortest incubation period prescribed*. As discussed earlier in this chapter, the maximum incubation period for the validation studies is actually the minimum incubation period for routine testing; standardized laboratory

cultures used in the challenge studies should be readily detected if test conditions are suitable for microbial recovery.

Prepare the Viability Test Group by plating the same volume of inoculum suspension used to challenge the Test Groups and Peptone Control Groups, using the pour-plate method or spread-plate method. In general, bacterial suspensions are plated with SCD agar and incubated at 30–35°C for 24–48 h. For fungi, use SDA medium and incubate the plates at 20–25°C for 3–5 d. This test is performed to confirm the low-level challenge required for the test. Ensure to observe mold plates on the third day of incubation to prevent inaccurate counts due to plate overgrowth.

To prepare a test-negative control, filter aliquots of the buffer diluents used in the test, remove the membrane filter and place it into a container with the same type of enrichment medium specified in the compendial method. Incubate the Test Groups, Peptone Control Groups, and the test-negative control as specified in the proposed test method, and proceed as directed in the compendial test for detection of specified microbial species. At the end of incubation period, observe broths for turbidity and selective media for characteristic growth. Compare the microbial recovery obtained from the Test Groups and the Peptone Control Groups. See Figure 7.6 for an example test diagram.

The test is valid if

- The challenge organism is recovered from the Peptone Control Groups.
- The Viability Groups confirm a low-level challenge (less than 100 CFU).
- The test-negative control shows absence of microbial contamination.

The proposed test method is suitable for the product being evaluated if the microbial growth recovered from the Test Group is comparable to the microbial growth obtained from the Peptone Control Group. Microbial growth is compared in appearance and biochemical reactions using selective media (if applicable).

Validation of TAMC and TYMC

The suitability testing for TAMC and TYMC should be performed using duplicate membrane filters for each challenge organism to account for variability in plate counts. For the Test Groups, each membrane filter is separately challenged with a low-level inoculum (less than 100 CFU) of a given challenge organism (see Figure 7.7). To prepare the Test Groups, dissolve/dilute 10 g or 10 mL of product into 90 mL of a suitable buffer diluent (1:10 sample dilution). Filter a 10-mL aliquot (equivalent to 1 g or mL of product) through a 0.45-µm membrane filter. *Note:* If screening for *Salmonella* spp., ensure that a minimum of 10 g or 10 mL of product is sampled. Then, rinse the membrane filter with at least two 100-mL portions of a chosen buffer (with or without inactivators), add the inoculum to the final rinse aliquot, and filter.

The Peptone Control Group is prepared by adding 10 mL of peptone water or buffer into 90 mL of the same buffer used to dissolve/dilute the product. A 10-mL aliquot of this buffer preparation is then filtered through a 0.45-µm membrane filter, and the membrane filter is rinsed with at least two 100-mL portions of the chosen

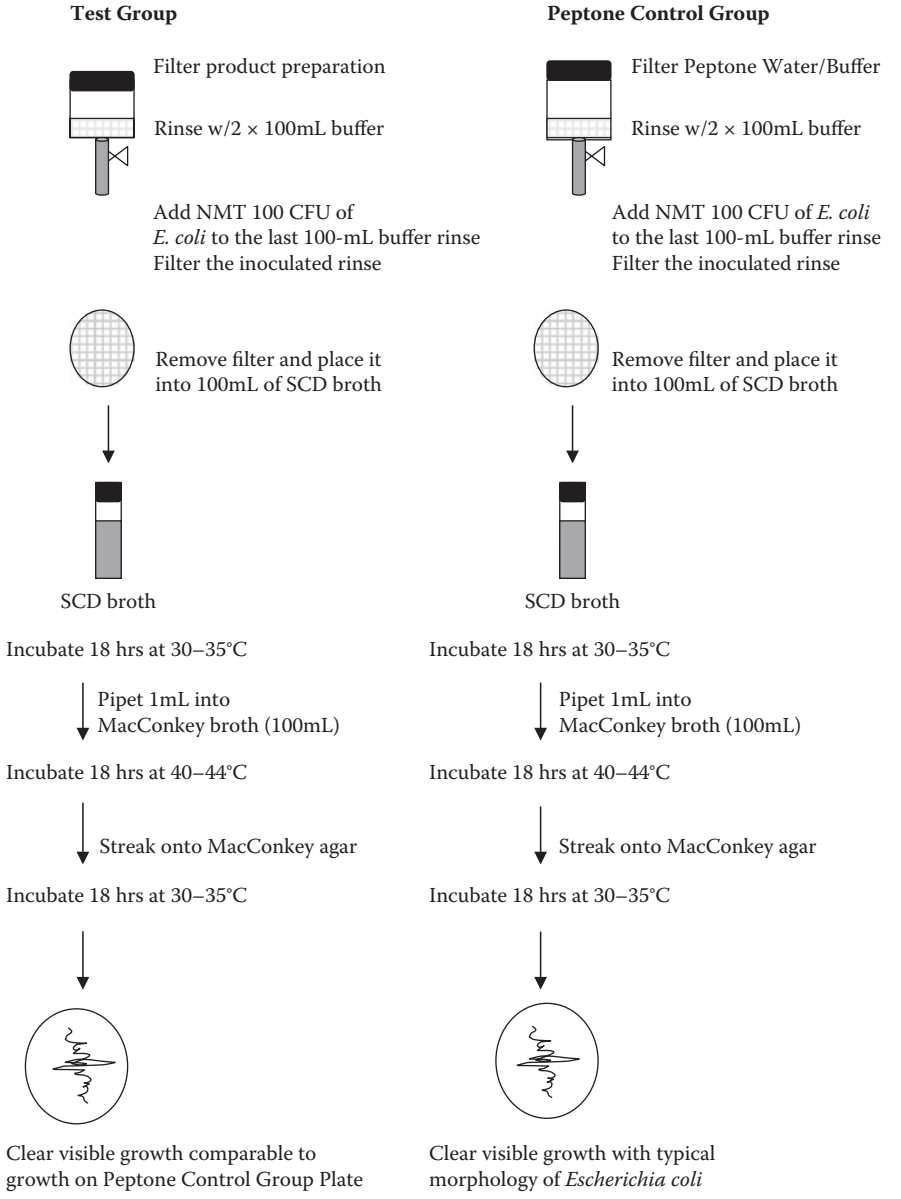


FIGURE 7.6 Suitability test for absence of *Escherichia coli* by membrane filtration.

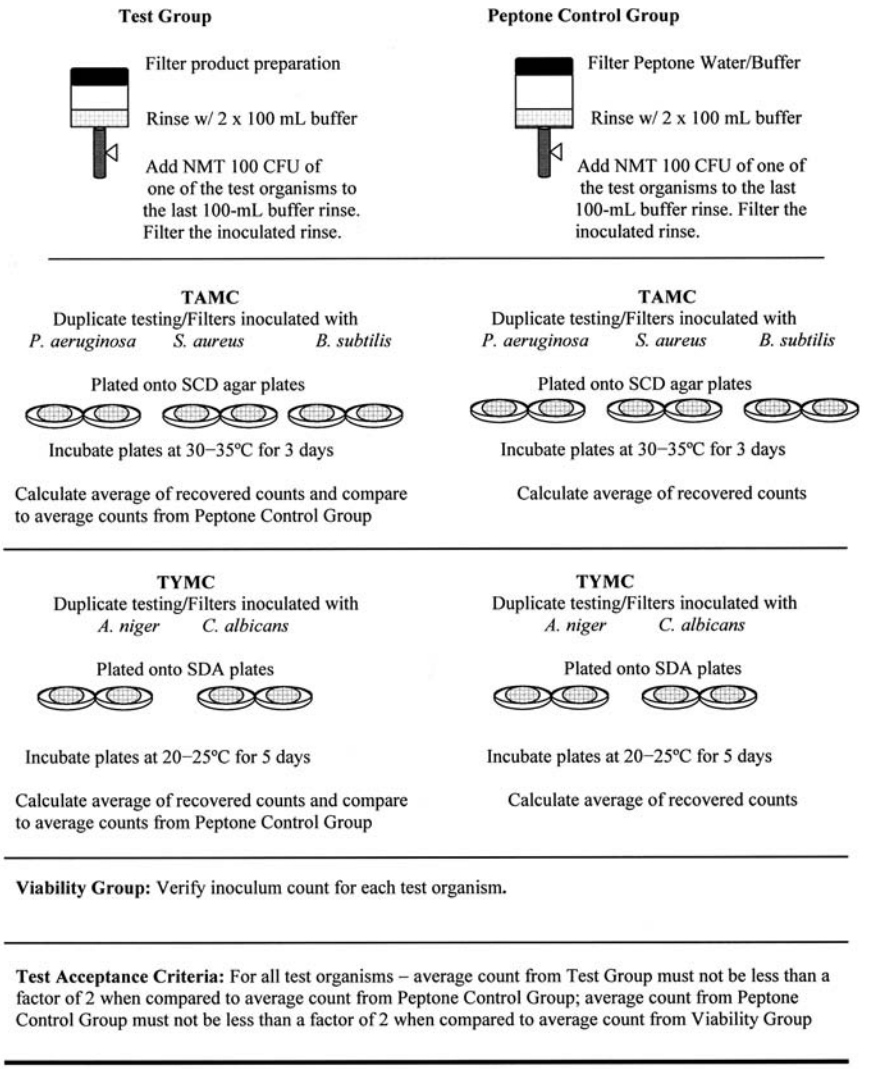


FIGURE 7.7 Suitability test for TAMC and TYMC by membrane filtration.

buffer, with or without inactivators. The low-level inoculum (less than 100 CFU) is then added to the final 100-mL rinse and filtered. This challenge is performed in duplicate, for each test organism.

For the TAMC challenge test, separately inoculate each final rinse with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Aspergillus niger,* and *Candida albicans*. For the TYMC challenge test, separately inoculate each duplicate final rinse with *Aspergillus niger* and *Candida albicans*. After the addition of the challenge organisms to the last rinse, remove the membrane filters and place them onto solidified media contained in Petri dishes. Use SCD agar plates for the TAMC test and SDA plates for the TYMC test.

To prepare a test-negative control, filter aliquots of the buffer diluents used in the test, in duplicate. Remove the two membrane filters and place one onto a solidified SCD medium plate and the other onto a solidified SDA plate. It is also recommended to prepare a product negative control, to evaluate any inherent product bioburden that could interfere with enumeration and evaluation of the recovered challenge organisms. A product negative control is prepared as described for the Test Group, but without the addition of test organisms.

Prepare the Viability Group by plating the same volume of organism suspension used to inoculate the Test Groups and Peptone Control Groups using the pour-plate method or spread-plate method. In general, bacterial suspensions are plated with SCD agar and incubated at 30–35°C for 24–48 h. For fungi, use SDA medium and incubate the plates at 20–25°C for 3–5 d. This test is performed to confirm the low-level challenge required for the test. Check mold plates after 3 d of incubation to prevent inaccurate counts due to plate overgrowth.

Incubate the TAMC plates at 30–35°C for NMT 3 d (USP states to incubate bacteria for NMT 3 d and fungi for NMT 5 d) and the TYMC plates at 20–25°C for NMT 5 d. As discussed earlier in this chapter, the maximum incubation period for the validation studies is actually the minimum incubation period for routine testing, because standardized laboratory cultures should be readily recovered under optimum test conditions.

At the end of incubation, enumerate the recovered colonies from the Test Group, Peptone Control Group, and Viability Group plates. Calculate the arithmetic mean number of colonies recovered on the replicate plates and report the results in whole CFU numbers. For each test organism, compare the mean value obtained for Test Group with the mean value obtained for the Peptone Control Group; also compare the mean value obtained for the Peptone Control Group with the mean value obtained for the Viability Group. If the mean count for the Test Group is not less than a factor of 2 when compared to that for the Peptone Control Group, the method shows adequate neutralizer effectiveness; if the mean count for the Peptone Control Group is not less than a factor of 2 when compared to that for the Viability Group, the method shows lack of neutralizer toxicity or interference with microbial recovery caused by the membrane filtration technique used (e.g., due to inherent toxicity of filter membrane, inadequate rinse, organism adherence to wall of filtration vessel, etc.).

The microbiologist must also evaluate the recovery obtained for the test-negative control and product negative control. For a valid test, the plates for the test-negative control should be free from microbial contamination. The plates for the product negative control should be free from microbial contamination or have a very low bioburden that will not interfere with the challenge studies.

Modifications to the Membrane Filtration Method

Failure to recover the test organisms or meet the test acceptance criteria under the test conditions necessitates some of the following modifications:

- Increase the number of rinses but do not exceed a washing cycle of 5 times for 200-mL rinse (reference USP Chapterv <71>, *Sterility Tests*).

- Incorporate a sufficient quantity of suitable inactivators in the rinse.
- Select an alternate type of membrane filter that is less toxic but still compatible to the product being tested.

If, following further testing attempts, no suitable neutralization method is found so that challenge organisms can be adequately recovered, it can be assumed that the inherent microbiocidal activity of the product will likely prevent contamination by the given microbial species. The additional data generated also provides assurance that the method suitability study was carried out using sound scientific principles and, therefore, the method is considered adequately challenged and validated. However, as it is possible that the spectrum of microbial inhibition is limited to the chosen test organisms, the manufacturing company should consider performing routine testing using the method that showed the best recovery or using the highest rinse volume attempted.

SUITABILITY OF MICROBIOLOGICAL MEDIA

The growth-promoting properties and sterility of the media used for method validation and for routine microbiological testing must be verified prior to use as part of the media quality control testing program in a microbiology laboratory. Detailed information on how to carry out this verification is included in the harmonized compendial chapters and will be summarized in the following text.

Microbiological media are divided into two basic categories: *selective* and *nutritive*. Selective media contain chemicals that inhibit the growth of certain types of microorganisms, thus selecting the growth of specific microbial species. Nutritive (nonselective) media are designed to promote the growth of a variety of bacteria and fungi.

According to the compendia, quality control testing should be performed for each batch of in-house–prepared or ready-prepared commercially available media. Tests to confirm media quality include pH, growth promotion, and growth inhibition (if applicable). It is acceptable to limit testing to each incoming lot of dehydrated medium if in-house–prepared media are sterilized using validated sterilization cycles. Otherwise, every batch of prepared media must be tested. Companies must also generate supporting documentation/data for the assigned media expiration dates. For commercially prepared media, a similar approach applies: if the vendor has been qualified through company-sponsored vendor audits and procedures are in place to control the shipment and storage of media from the vendor site to the user site, reduced quality control testing at the user site may be acceptable.

The need to retest commercially prepared media has been debated extensively throughout the industry and most companies now believe that this additional testing, which imposes a significant financial burden on the user, is not necessary for media that are of proven reliability. In the United States, commercially prepared media are listed as medical devices and thus regulated accordingly by the Department of Health and Human Services and the FDA. Therefore, media manufacturers perform extensive quality control testing at their sites, and certificates of analysis for each batch of medium produced are issued and made available to their customers.

The decision to perform limited testing at the user site must lie with each company and may need to be made on a case-by-case basis, depending on the type and use of the media and on the vendor. Because most companies purchase media from reliable sources, users may be able to justify a reduced testing program at the user sites if they generate sufficient data (usually during a one-year period) to demonstrate the growth promotion properties of incoming media. Once sufficient data have been collected for statistical analysis, the user may be able to justify reduced testing for media types that are of proven reliability.

The National Committee on Clinical Laboratory Standards (NCCLS) *Quality Control for Commercially Prepared Microbiological Culture Media* Document M22-A3 [1] recommends quality control testing for each batch of medium that has a failure rate > 0.5%. For media with a failure rate <0.5%, limited testing (e.g., the first three incoming lots in a one-year period) to no in-house testing is suggested. This standard was discussed in an article published in the USP *Pharmacopeial Forum* in which the author points out the facts that media manufacturers do follow the NCCLS Standard M22-A3 and that most media used in clinical microbiology is not subjected to routine growth-promoting tests by the users [2].

In general, most commercially available media maintain their growth-supporting capabilities as long as they are stored according to the manufacturer's recommendations. Only the media that are more fastidious have demonstrated a greater growth promotion failure rate. According to the NCCLS, M22-A3 2001 media survey, some examples of media that have demonstrated high extrapolated failure rates (EFR) include BHI agar w/sheep blood w/cyclohexidine and chloramphenicol (EFR = 0.74), Cornmeal agar w/Tween (EFR = 1.34), MacConkey w/sorbitol (EFR = 0.61), and nutrient broth (EFR = 1.32). In fact, if there is one concern that a customer should have, that is the environmental and physical conditions during media shipment as less than adequate storage conditions can contribute to quality control testing failure of the media. If manufacturers can ensure the control of environmental conditions during shipment and if this information is made available to customers, it can serve as additional proof to support limited testing.

Although media growth promotion and pH testing may be reduced, the user should always inspect the media containers upon arrival for signs of breach of container integrity or adverse conditions during shipment. The visual observation should include inspection for problems such as cracked containers, frozen or melted agar, insufficient agar in plates or unequal filling in containers, change in expected color, excessive bubbles, presence of precipitates, and obvious microbial contamination. Suspect media, damaged containers, and contaminated media must be discarded, documented, and reported to the vendor.

The media quality control program should also include a *sterility check* to detect for adventitious contamination during shipment not detected by visual inspection. The user may refer to USP Chapter <71> for a guideline as to the number of media containers to be incubated for the sterility check procedure. For example, for shipments with less than 100 articles, the user may incubate the equivalent of 10% of the total number of units or 4 units, whichever is less; for shipments with more than 100 but less than 500 articles, the user may incubate 10 units; for shipments with more than 500 articles, the user may incubate the equivalent of 2% of the total number of units or 20 units, whichever is less.

To perform a sterility check (detection of gross contamination), the author recommends incubating the media articles at 30–35°C for not less than 3 d. Some companies prefer to preincubate most, if not all, media articles for a short period of time to ensure that no sporadic adventitious contamination will interfere with routine testing. In Chapter <1117>, the USP recommends special care for media used in environmental monitoring of critical areas: for media articles that are not double wrapped and terminally sterilized, the USP recommends preincubation and 100% inspection prior to use. Regardless of whether media are preincubated or not, prior to starting a test, microbiologists should always perform a 100% inspection of media containers. Any suspect media container must be discarded and not used in the test.

Growth Promotion Testing for Microbial Enumeration Media

For verification of growth promoting properties of media used for bioburden determination, the medium is challenged via direct plating (pour plate or spread plate) with a low-level inoculum (less than 100 CFU) of a given test organism and incubated as used in the test. See the section "Preparation of Working Cultures" for directions on standardization of inocula. As per the compendia, the counts obtained must not differ by more than a factor of 2 from the calculated value for the inoculum used. In addition, if using a freshly prepared inoculum, growth obtained with the new medium must be comparable to the growth obtained using a medium that had been previously tested and approved, which was tested concurrently with the new medium, with respect to typical colony size and morphology. For liquid media, verification of adequate growth promoting properties occurs if, after inoculation and incubation, microbial growth is copious, clearly visible, and comparable to a previously tested and approved media batch.

SCD medium (and another all-purpose growth medium) is challenged with *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Aspergillus niger*, and *Candida albicans*. Bacterial preparations incubate at 30–35°C for NMT 3 d and fungal preparations at 30–35°C for NMT 5 d. Mold plates should be checked following a 3-day incubation to avoid inaccurate counts due to plate overgrowth. Growth promotion of SDA medium (or another fungal medium) is performed with *Aspergillus niger* and *Candida albicans*, incubating at 20–25°C for NMT 5 d. It is recommended that the media used for microbial enumeration tests be challenged also with representative environmental isolates and specified strains of concern, especially if those will be included in the method suitability testing.

Growth Promotion Testing for Selective Media

Media used in the screening tests for selected microbial species must be evaluated for growth promoting as well as indicative and/or inhibitory properties using a low-level inoculum (less than 100 CFU) of specified test organisms. See Table 7.10 for information on test strains. Additional microorganisms may be used as deemed appropriate.

For testing of *liquid media for growth-promoting properties*, inoculate and incubate the new lot alongside a previously tested and approved lot at the temperature given, and *for not more than the shortest period of time* specified, in the compendial

TABLE 7.10
Test Organisms for Verification of Nutritive, Inhibitory, and Indicative Properties

Medium	Growth Promotion (Nutritive Property) Test Organisms	Inhibitory Property Test Organisms	Indicative Property Test Organisms
Enterobacteria enrichment broth-Mossel (MEEB)	*E. coli and P. aeruginosa*	*S. aureus*	
Violet red bile glucose agar (VRBGA)	*E. coli* and *P. aeruginosa*		*E. coli* and *P. aeruginosa*
MacConkey broth	*E. coli*	*S. aureus*	
MacConkey agar	*E. coli*		*E. coli*
Rappaport Vassiliadis *Salmonella* enrichment broth (RVSEB)	*S. enterica* ssp. *enterica*—serotype typhimurium or abony	*S. aureus*	
Xylose, lysine, deoxycholate agar (XLD)	*S. enterica* ssp. *enterica*—serotype typhimurium or abony		*S. enterica* ssp. *enterica*—serotype typhimurium or abony, and *E. coli*
Cetrimide agar	*P. aeruginosa*	*E. coli*	
Mannitol salt agar (MSA)	*S. aureus*	*E. coli*	*S. aureus*
Reinforced medium for clostridia (RMC)	*C. sporogenes*		
Columbia agar (CAM)	*C. sporogenes*		
Sabouraud dextrose broth (test for *C. albicans* only)	*C. albicans*		
Sabouraud dextrose agar (test for *C. albicans* only)	*C. albicans*		*C. albicans*

test. Microbial growth observed for the new lot should be copious, clearly visible, and comparable to the growth from the previously tested lot.

For testing of *solid media for growth-promoting properties*, inoculate and incubate a plate or tube of the new lot alongside a previously tested and approved lot at the temperature given, and *for not more than the shortest period of time* specified, in the compendial test. Microbial growth observed for the new lot should be comparable to the growth from the previously tested lot.

To test for *indicative properties*, inoculate and incubate a plate or tube of the new lot alongside a previously tested and approved lot at the temperature given, and *for a time period within the range* specified, in the compendial test. Microbial growth observed for the new lot should be comparable to the growth from the previously tested lot in terms of morphology and indicative biochemical reactions.

To test for *inhibitory properties*, inoculate and incubate a plate or tube of the new lot at the temperature given, and *for not less than the longest time period* specified, in the compendial test. No microbial growth should occur in this case.

VALIDATION OF RAPID MICROBIOLOGICAL METHODS

Rapid microbiological methods (RMM) require validation prior to implementation, and in this section, the author will address validation of alternative technologies for microbial detection (qualitative method), enumeration (quantitative method), and identification. As many of the RMM techniques used in medical microbiology and in the food industry are not suitable for application in the pharmaceutical industry, companies must create their own validation protocols to demonstrate that the chosen method is appropriate for the application and generates reproducible results. Validation documents need to address the theoretical basis for the test as applied to the type of sample to be tested. The user also needs to define which test parameters will be challenged in order to demonstrate that the alternative method is equivalent or better when compared to the compendial test. Guidance on the use of alternate methods is provided in the USP *General Notices* Section *Tests and Assays,* and in the EP *General Notices* Section 1.1. These compendial documents point out that in the case of a dispute, only the result obtained using the compendial test is considered conclusive. Therefore, it is imperative that scientifically sound validation packages be prepared so that companies can receive regulatory approval for the alternate methods and can defend the data generated using those methods.

There are three main guidance documents for validation of RMM and they are USP31-NF26 Chapter <1223>, *Validation of Alternative Microbiological Methods*; the EP 6.0 Chapter 5.1.6, *Alternative Methods for Control of Microbiological Quality;* and the PDA Technical Report No. 33, *Evaluation, Validation and Implementation of New Microbiological Testing Methods* [3]. These documents, which contain detailed recommendation for validation strategies, can be used as a guide when creating a validation protocol for the chosen alternative method.

The Validation Package

Performing a validation for a new or alternate method involves more than just generating test results. The validation protocol execution covers various stages of method and equipment suitability testing designed to demonstrate that the new system meets the requirements for its intended application, and shows that when compared to the compendial method, the alternate system is equivalent or better. Included in the test protocol are challenges to the performance of equipment, both hardware and software, as well as criteria for qualification of the testing laboratory and technicians.

A company should start the process of implementation of the new alternate method with a design phase, in which the evaluation of method appropriateness for the intended application is considered. During this phase, the company should document the justifications for the change and define the user's requirements and design specifications for the new equipment; candidate systems are often selected based on capability for sample throughput, method sensitivity and specificity, needed skill level for operators, and data management capabilities. Once the type of system has been chosen, the company needs to start the process of vendor selection, taking into consideration economic benefits, vendor support services, and regulatory requirements. It may be worthwhile to test a loaned or rented system for verification of proof-of-concept prior to purchase. This prevalidation activity may

save the company money and resources in case the new technology does not deliver the expected results.

The steps that follow are very similar to the ones described for equipment validation and which comprise of three phases: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). Most vendors of automated rapid microbiology systems can supply the users with validation packages, and many offer assistance with the IQ activities. During IQ, the company ensures that the equipment received meets the user's requirements and that the system has been properly installed according to its specifications. During OQ, the company verifies and documents that the installed system operates within predetermined limits in its laboratory environment. This phase can be considered a confirmation of proof-of-concept, and it serves to establish the method's limits and tolerances. During the OQ phase, the hardware and software are also validated using an in-house or a vendor-supplied protocol.

The last phase of validation is the PQ. It generates sufficient data to document that the system consistently performs as expected within predetermined criteria for the testing of product samples. During PQ, the system is challenged multiple times, typically using at least two analysts, to ensure that results are consistent and reliable over time. Prior to the conclusion of PQ, the company should create applicable standard operating procedures, including equipment operation and maintenance and change control. Training qualification of personnel involved with the validation work must also be documented prior to closing the protocol.

Validation Criteria

The criteria for validation of microbial methods vary, depending on whether the test is designed for qualitative, quantitative, or identification purposes. Table 7.11 (adapted from USP Chapter <1223>, Table 1) describes the typical validation parameters based on the type of test. Equivalency is demonstrated via testing inoculated

TABLE 7.11
Method Validation Parameters

Parameter	Quantitative Test (Microbial Enumeration)	Qualitative Test (Presence/Absence)	Microbial Identification
Accuracy	✓	EP recommended	✓
Precision	✓	EP recommended	✓
Specificity	✓	✓	
Limit of detection (LOD)	✓	✓	
Limit of quantitation (LOQ)	✓		
Linearity	✓		
Operating range	✓		
Robustness	✓	✓	✓
Ruggedness/repeatability/ reproducibility	✓	✓	✓

samples by the compendial and the new methods. Companies must be careful when designing their testing protocols because some RMM systems can detect a significantly greater number of organisms when compared to traditional methods, especially the ones that do not rely on cell viability for detection. In addition, testing protocols must take into consideration the large degree of variability typical of traditional microbiological methods.

Validation of Quantitative Methods

Most microbial quantitation methods involve the enumeration of microbial colonies, which follow a Poisson distribution. Therefore, the USP recommends the use of statistical tools appropriate to the Poisson curve when analyzing quantitative data. However, many microbiologists prefer to use tools that are applicable to normally distributed data, thus using the $\log_{10}$ transformation of the raw counts. This approach is also acceptable to the USP, for which currently normal plate count variability is defined as 0.5-log variation in Chapter <51> Antimicrobial Effectiveness Testing and in Chapter <1227>.

Accuracy

Accuracy is defined by how close the results obtained by the alternative method are to those generated by the compendial method. This test parameter is usually expressed as the percentage recovery of the total inoculum used to spike the test samples, and it should be demonstrated across the operating range of the test method. For plate count methods, the testing range should be 25–250 CFU. At least five inoculum levels, using various microbial species, should be used to challenge the new system. This can be accomplished by preparing an inoculum suspension at the upper end of the operating range of the assay, and diluting five levels down to the lower end of the range (e.g., 100%, 80%, 60%, 40%, 20%, and 10%). Testing should be performed with inoculated product and a buffer solution (control) in order to verify any potential product interference with microbial recovery when using the new technology. For the new method to satisfy this validation parameter, recovery of viable organisms must be equal to or better than the recovery obtained with the traditional method. Results can be calculated using statistical analysis (e.g., analysis of variance [ANOVA]) or by simply calculating percent recoveries (recommended: ± 30% recovery variability when compared to compendial method).

Specificity

This parameter is defined as the ability of the method to detect different types of microorganisms under various metabolic conditions. During this challenge, it will be possible to verify whether the new technology is capable of detecting viable but nonculturable organisms (VNCs), an added specificity that could impact established product specifications. Therefore, including challenges to encourage false positive results (e.g., detection of organisms that would not produce a positive result with a traditional method) becomes an important validation parameter. Testing for specificity can be accomplished by screening various samples inoculated with a wide range

of microbial species (inoculated product and controls). The new method meets the specificity test requirement if all challenge microorganisms are successfully isolated and enumerated. If the new technology is proven better than the traditional method, a thorough assessment of potential negative impact to existing established product limits must be performed and appropriate measures taken to address the findings.

Precision

Precision, usually expressed as the standard deviation (SD) or percent relative standard deviation (percent RSD), is defined as the degree of agreement among individual multiple test results. Microbial enumeration procedures are known to have very large variability in comparison to chemical methods. Therefore, the more replicates tested, the more precise the microbial count will be. For this test, at least 10 replicates of each of five inoculum levels, covering the range of the test, should be prepared. Ensure that the product and control preparations are challenged with representative microbial species. In general, a coefficient of variation not to exceed 30% is acceptable. *Note:* The EP states that a coefficient of variation in the range of 10–15% is an acceptable target precision value. The new method is deemed equivalent if the calculated coefficient of variation is not greater than the one obtained with the traditional method.

Limit of Quantitation

This parameter defines the lowest level of microorganisms that can be counted with acceptable precision and accuracy. To perform this test, at least five replicates of each of five different levels of inoculum, from each type of test organism, are prepared. As discussed above, the chosen inoculum preparations should cover the entire operating range of the assay. Product and control samples are separately inoculated with the various inoculum challenge levels, and processed using both the compendial and the new methods. To be deemed acceptable, the new method should not produce a value for the limit of quantitation greater than the one obtained when using the traditional compendial method.

Linearity

In a microbial enumeration test, linearity is defined as the ability of the method to yield results that are proportional to the concentration of organisms within the specified operating range of the assay. In order to test for this parameter, the microbiologist should prepare at least five inoculum concentrations of each test organism and perform at least five replicate challenges for each organism. Once the microbial counts are tabulated, a linear regression analysis is performed to determine the correlation coefficient (r^2) value of the best-fit curve. According to the USP, the alternative method shows acceptable linearity if the calculated r^2 is greater than 0.95.

Limit of Detection

This test parameter determines the lowest number of microorganisms that can be detected in a sample, but not necessarily enumerated with accuracy or precision. This is a measure of test sensitivity. To test this parameter for a quantitative assay, the micro-

biologist should inoculate at least five replicates of product and control samples with a very low level inoculum of the test organisms (not more than 5 CFU). The inoculum level may need to be adjusted to ensure that at least 50% of the samples yield microbial growth when tested using the compendial method. Samples should not be diluted or incubated as limit of detection refers to the bioburden in the original material.

Following inoculation, the bioload added to the test samples is measured using both the compendial and the new method. Limit of detection can either be determined using the chi-square test or the most probable number (MPN) technique. When using the MPN technique, the methods are considered equivalent if the 95% confidence intervals overlap. The chi-square test is used as the goodness-to-fit test, that is, for estimating how closely an observed distribution (new method) matches an expected distribution (compendial method). Note that the limit of detection is affected by the amount of product and sample dilution used. For example, if a sample amount equal to 10 g is tested using a 1:100 dilution and 1-mL aliquots are plated, the absence of microbial recovery on the test plates would be reported as <100 CFU/g. However, if 5-mL aliquots of this same sample preparation were plated, the absence of microbial recovery would be reported as <20 CFU/g.

Range

For quantitative methods, the operational range of the method is the interval between the lowest and highest inoculum levels that can be enumerated with precision, accuracy, and linearity. When testing various types of organisms, the acceptable range most likely will be different for bacteria and fungi. As discussed earlier, for traditional microbiological plate count methods, an acceptable range for bacteria and yeast counts is 25–250 CFU and for mold the range is 8–80 CFU. An alternative technology may in fact be able to extend the operating range of the assay for both the upper and lower limits.

Ruggedness

Ruggedness is a validation parameter that measures the degree of reproducibility of the method under various operational and environmental conditions. This is not necessarily a test to compare the new method with an existing one. Typical variables tested to verify this parameter include different analysts; different instruments; and different lots of reagents, test supplies, and media. Ruggedness is often best determined by the vendor. In fact, the PDA Technical Report No. 33 states that "data supplied by the test method manufacturer are entirely admissible to prove validation of ruggedness" [3]. However, the author believes that the user should show due diligence and test at least some of the variables mentioned above. To perform this test, a minimum of five replicates of the experiment should be performed for each test variable. In general, a coefficient of variation not exceeding 15% is acceptable.

Robustness

Robustness measures the degree up to which a method is able to remain unaffected by small changes in method parameters. As with ruggedness, robustness is not necessar-

ily a comparison between two methods and it is best carried out by the manufacturer of the equipment. However, it is important for the user to perform at least a few challenges to determine the optimum operating parameters of the new system in the laboratory setting. The user should consult with the vendor as to an appropriate testing protocol to be used, especially if critical parameters are modified to best meet the user's requirements. For some systems, no testing at the user site may be needed.

Validation of Qualitative Methods

Qualitative methods are tests for microbial contamination detection verified by the presence or absence of turbidity in liquid media. When validating new methodologies for qualitative procedures, some validation parameters discussed previously also apply.

Specificity

The test for specificity is especially critical for alternative microbiological methods that are not growth-based technologies. Testing for this parameter will ensure that extraneous nonmicrobial particles will not interfere with the test method. The test protocol is similar to the one described for quantitative methods, and it involves screening the new method against specific microbial strains. The new method meets the specificity requirement if all challenge microorganisms are successfully detected. If the new technology is proven better than the traditional method, a thorough assessment of potential negative impact to existing established product limits must be performed and appropriate measures taken to address the finding.

Limit of Detection

This test parameter determines the lowest number of microorganisms that can be detected in a sample, but not necessarily enumerated. As described previously, the limit of detection is determined by inoculating various samples with a very low-level inoculum (not more than 5 CFU) of specified test organisms. Remember that this validation parameter must be tested prior to sample dilution or incubation, and in this case, only presence of the test organism is required.

Ruggedness

Ruggedness should be tested for qualitative alternate methods and, as discussed earlier, this validation parameter is verified extensively by equipment vendors. However, as in the case with quantitative methods, the user should be diligent enough to challenge at least some variables such as different analysts, different media/reagents, and different lots of test supplies.

Robustness

Robustness should also be determined for qualitative methods, and this validation parameter is often best verified by the equipment vendor. However, the user should consult with the vendor as to the need for further testing at the user site.

Accuracy and Precision

Although not included in the USP Chapter <1223> as these two validation parameters are not typically tested for qualitative assays, the EP does consider accuracy and precision when demonstrating equivalency between two qualitative methods. According to the EP, the user should perform side-by-side low-level challenges using the new and the compendial methods, and determine the relative rate of false positive and false negative results.

Validation of Automated Microbial Identification Methods

Microbial identification methods vary greatly in their outputs. Therefore, the user should not attempt to compare one system's performance against another but, rather, should focus on the capabilities and application of the new technology and ensure that the system provides consistent results over time. For microbial identification systems, a typical validation protocol includes challenges to the hardware and software, and performance verification using microorganisms from standard culture collections as well as environmental isolates.

Accuracy

This validation parameter demonstrates the ability of the system to identify specified organisms to the required taxonomic level. Challenges to verify accuracy are performed using a broad range of microorganisms, including test strains recommended by the vendor and others that are of interest (or concern) to the user. Typically, the user tests three unique suspensions of each chosen organism. The results obtained are compared to the known identification. When performing challenges to verify accuracy, the user should consider testing for false positives and false negatives, especially for systems that are based on phenotypic profiling. For example, the system must not provide identification results for mixed samples and it should alert the user as to the need to prepare a pure culture for the test. If discrepancies exist, the user should investigate the findings for an assessment of possible limitations of the automated system.

Precision

This validation parameter determines the degree of agreement among individual identification results when multiple samples of the same microbial suspension are tested repeatedly over time.

Robustness

This validation parameter is often determined by the vendor. However, users may find it appropriate to confirm some of the challenge tests in their testing environment.

Ruggedness

As with robustness, ruggedness is a key validation parameter that is often tested by the vendor. However, users should consider confirming some of the challenges in their testing environment, and those include performing the validation using separate analysts and different lots of media, reagents, and test supplies.

Final Thoughts

Validation of alternative methods should be performed against the compendial referee methods. Until the harmonized microbial limit test methods are made official, companies should validate new methods against the current official methods. According to the EP, if an alternative method has already been validated against a current compendial method and approved by the regulatory agencies, no revalidation work is required once the harmonized chapters become official.

One final note about the validation of alternative microbiological methods: each new technology has its own application and limitations, and each offers unique validation challenges. Therefore, the user must be careful not to create a single protocol for the validation of a new technology platform that will be used as a substitute to more than one compendial method. For example, using an alternate microbial enumeration system for detection of bioburden in raw materials and in purified water systems will require separate protocols with different challenge tests; although in principle both samples are evaluated using similar traditional plate count procedures, using a rapid microbiological method, the user will certainly face some unique challenges. For example, raw materials, especially the ones from natural sources, typically have a high bioload whereas purified water is expected to have little to no counts; raw materials may interfere with the method detection system when no interference should be expected for water samples.

In summary, although validation of alternative microbiological methods offers manufacturers the opportunity to gain efficiency, test sensitivity, and convenience, companies must take the time to carefully evaluate the available technologies against their user's requirements to fully appreciate the alternate system's capabilities, limitations, and, above all, to create validation protocols that will withstand the scrutiny of regulators and fully support the method change.

POINTS TO CONSIDER

- Create a standard protocol for method suitability studies, specifying parameters, acceptance criteria, and maximum dilutions to be attempted, prior to initiating the work.
- For membrane filtration methods, ensure that membrane filters compatible to the product being tested are chosen. Once the method is validated with a particular type of membrane, using an alternate membrane during routine testing is considered a change to the validated method.
- For the pour-plate method, 100 × 15 mm petri dishes and 15–20 mL of agar medium are typically used. If larger petri dishes are employed, the volume of the medium used must be increased accordingly.
- All test conditions (i.e., sample preparation, reagents, incubation, etc.) should be standardized and performed in the method suitability work as performed in the proposed actual test.
- The method for sample preparation depends on the physical properties of the product being tested. If the compendial method is not suitable for a given product, an alternative method may be developed.

- Optimal conditions for microbial growth must be present to ensure adequate recovery and reproducible test results.
- Proportionally larger or smaller quantities than the specified weights and volumes of the test substances may be used for method suitability studies, provided that the measurement is made with at least equivalent accuracy and that dilutions are adjusted accordingly to yield equivalent concentrations compared to the compendial methods.
- When developing a procedure for sample preparation, consider the use of strict aseptic procedures and attempt to minimize steps to prevent potential adventitious contamination during routine testing.
- Filtration alone may not remove antimicrobial agents. Some chemicals and products (e.g., benzalkonium chloride) tend to bind to certain types of filter, leaving a bactericidal residue on the surface of the membrane. In these cases, additional rinses and/or use of low-binding membrane filters, such as polyvinylidine difluoride (PVDF), may enhance microbial recovery.
- In choosing a neutralizer, one must evaluate its efficacy, product compatibility, and microbial toxicity.
- It is important to add representative environmental isolates to the challenge studies.
- It may be necessary to use alternate media and different incubation conditions in order to adequately recover some environmental strains.
- If a validated product undergoes a chemical or physical change or if a change is introduced to the validated test, the method suitability study must be repeated.
- Validation of the MPN procedure is accomplished by inoculating the sample preparation with low-level inocula of the proposed challenge organisms used in the TAMC test and then carrying out the procedure. Perform positive controls by inoculating the chosen diluent and applying the MPN method after omitting the sample. A Viability Group to verify inoculum challenge and test-negative controls should also be prepared. The method is considered valid if the calculated value from the inoculum is within 95% confidence limits of the results obtained for the Test Group.
- Although the compendial microbial examination tests have been harmonized, there are still some discrepancies among some of the existing USP microbiology chapters, and those must be corrected. For example, the USP informational Chapter <1227> recommends a microbial recovery of not less than 70% for the microbial enumeration tests while the harmonized Chapter <61> requires that counts not differ by more than a factor of 2 from the inoculum challenge. USP Chapters <1227> and <51> define no change in microbial counts (error variance) as 0.5-log variability (0.5 log units). In the harmonized Chapter <61>, acceptable microbial variability is defined as a factor of 2 (0.3 log). Based on recent information obtained from USP representatives, there is an initiative to harmonize the USP chapters and to eliminate any conflicting or contradicting information. Until then, for the purpose of validating compendial microbial limit tests, microbiologists should refer to the harmonized referee chapters.

For noncompendial methods, using 0.5 log should still be considered an acceptable approach to define normal plate count variability.

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8 Microbiological Quality of Pharmaceutical and Biopharmaceutical Products and Raw Materials

The microbiological quality of nonsterile finished products, in-process formulations, and raw materials must be controlled and monitored against established specifications to ensure quality, safety, and therapeutic activity of the drug product manufactured. Although the manufacturing process and microbiological quality of active pharmaceutical ingredients is highly controlled and must comply with current good manufacturing practices (cGMPs), many excipients used in the production of a drug product may come from suppliers whose primary customers are not pharmaceutical companies. This fact can be of concern because the microbial quality of raw materials may negatively impact the quality of the drug product manufactured. In this chapter, the author will address compendial and industry standards for the microbiological quality of pharmaceutical articles with focus on global harmonization efforts.

MICROBIOLOGICAL TESTING

The microbiological quality of pharmaceutical/biopharmaceutical products and raw materials is determined using test methods that quantify the bioload in the substance. In addition, testing for specified microbial species is performed if these types of organisms are deemed objectionable to the production process or the final product manufactured.

Raw Materials

Raw materials used in pharmaceutical manufacturing include active pharmaceutical ingredients and excipients. An active pharmaceutical ingredient (API) is the substance in the drug formulation that is therapeutically active. Excipients are raw materials included in a drug formulation to improve its physical qualities, such as a drug delivery system. Pharmaceutical excipients are classified by the function they perform in a drug formulation, and these include fillers, flavors, colors, lubricants, preservatives, coatings, disintegrants, and binders. Raw materials are rarely sterile, and some may need to undergo special treatment to render them microbiologically acceptable for use.

These ingredients can be of natural (plant, animal, or mineral) or chemical (synthetic or semisynthetic) origin, which in itself will determine the likelihood of the substance having a high bioload or harboring objectionable organisms. Water, being one of the most common raw materials used in pharmaceutical manufacturing, is specially treated, purified, and carefully monitored, as discussed in Chapter 4.

For APIs, standards for good manufacturing practices are in place. However, currently there are no clear regulatory standards for good manufacturing practices for excipients in the United States, Europe, India, or China, the latter two countries being the main providers of raw materials to drug manufacturers in Europe and the United States. In an attempt to address this issue, the International Pharmaceutical Excipients Council (IPEC) is in the process of launching a guide for GMP production of excipients, one that would meet the needs of pharmaceutical manufacturers around the world.

Regardless of the current regulatory status of excipient manufacturers, all raw materials used in pharmaceutical production must be qualified according to regulatory standards—companies must ensure that suppliers are audited against established cGMP regulations and that appropriate testing, to include bioburden, testing for specified microorganisms, and bacterial endotoxin (where applicable) is carried out either by the supplier, pharmaceutical company, or both.

In the United States, standards for quality, purity, identity, and/or strength of raw materials are established by the committees of the USP Convention Inc., an independent body that publishes and maintains the USP, National Formulary (NF), and USP Reference Standards. For substances that have FDA-approved use as drugs, standards are published in USP monographs. Standards for excipients, vitamins, minerals, botanicals, or herbal substances are published in the NF. The USP-NF–published standards are recognized as official, and the FDA and state government agencies can enforce them to assure that pharmaceutical products marketed in the United States are manufactured in full compliance with cGMPs. Similar quality standards for raw materials are also present in other pharmacopeias, including the European Pharmacopeia (EP) and the Japanese Pharmacopeia (JP).

Many compendial monographs for raw materials have specifications for microbiological quality. Table 8.1 provides a list of the most common excipients used in pharmaceutical production in the United States and their applicable global microbiological acceptance criteria.

Biopharmaceutical Products

In biopharmaceutical production, the front end of the drug manufacturing process is carried out in an axenic manner to ensure that the fermentation culture is free from viable organisms other than the host cells (in case of microbial fermentations). As such, media, buffers, and other components, as well as equipment used in the production are either heat or filter sterilized. Although testing for detection of adventitious contamination in fermentation and cell culture samples (i.e., nonhost contamination) is a regulatory requirement, there are no compendial tests or regulatory guidance documents addressing this topic. Hence, most companies have validated in-house methods based on compendial microbial limit testing using various selective and nonselective media and incubation conditions. However, method harmonization/

TABLE 8.1
Common Excipients Used in U.S. Pharmaceutical Production

Excipient	Pharmaceutical Use	Source/Manufacturing Process	Microbial Specifications	
			USP31-NF26	Ph.Eur. (EP) 6.0 (2008)
Calcium phosphate (dibasic)	Buffering and sequestering agent	Natural—anhydrous material	None	None
Calcium stearate	Tablet and capsule lubricant	Chemical—reaction of calcium chloride with sodium salts of stearic and palmitic acids	None	TAMC: NMT 10^3 CFU/g; absence of *E. coli*
Croscarmellose sodium	Tablet and capsule disintegrant	Natural—steeping of cellulose in sodium hydroxide solution	TAMC: NMT 10^3 CFU/g; TYMC: NMT 10^2 CFU/g; absence of *E. coli*	TAMC: NMT 10^3 bacteria/g; TYMC: NMT 10^2 CFU/g; absence of *E. coli*
Crospovidone	Tablet disintegrant	Chemical—"popcorn polymerization" process	None	None
Ethylcellulose	Flavoring fixative; tablet binder and filler; coating and viscosity-increasing agent	Natural—chemical process for purification and ethylation of cellulose	None	None
Gelatin	Coating, suspending and gelling agent; tablet binder; viscosity-increasing agent	Natural—extracted from animal tissue	TAMC: NMT 10^3 CFU/g; absence of *Salmonella* spp. and *E. coli*	TAMC: NMT 10^3 CFU/g; absence of *Salmonella* spp. and *E. coli*
Hydroxypropyl cellulose	Coating, stabilizing, viscosity-increasing and suspending agent; tablet binder	Natural—chemical process for purification and etherification of cellulose	None	None

Continued

TABLE 8.1 (Continued)
Common Excipients Used in U.S. Pharmaceutical Production

Excipient	Pharmaceutical Use	Source/Manufacturing Process	Microbial Specifications	
			USP31-NF26	Ph.Eur. (EP) 6.0 (2008)
Hydroxypropyl methylcellulose (Hypromellose)	Coating and stabilizing agent; film-former; rate-controlling agent for sustained release; tablet binder; viscosity-increasing agent	Chemical—reaction of purified alkali cellulose with chloromethane and propylene oxide	None	None
Lactose, anhydrous	Binding and tableting agent; lyophilization aid; tablet and capsule filler	Natural—plant origin; produced by roller drying solution of lactose above 93.5°C	None	None
Lactose, monohydrate	Tablet binder; tablet, capsule, and dry-power inhaler diluent	Natural—disaccharide of galactose, and glucose present in milk of most mammals	TAMC: NMT 10^2 CFU/g; TYMC: NMT 50 CFU/g; absence of *E. coli*	TAMC: NMT 10^2 CFU/g; absence of *E. coli*
Magnesium stearate	Tablet and capsule lubricant	Chemical—reaction of magnesium chloride with sodium stearate or magnesium oxide, hydroxide, or carbonate with stearic acid at elevated temperatures	TAMC: NMT 10^3 CFU/g; TYMC: NMT 500 CFU/g; absence of *Salmonella* spp. and *E. coli*	TAMC: NMT 10^3 CFU/g; absence of *E. coli*
Microcrystalline cellulose	Adsorbent and suspending agent; tablet and capsule diluent; tablet disintegrant	Natural—hydrolysis with dilute mineral acid solutions of α-cellulose followed by purification by filtration and drying	TAMC: NMT 10^3 CFU/g; TYMC: NMT 10^2 CFU/g; absence of *S. aureus*, *P. aeruginosa*, *Salmonella* spp., and *E. coli*	TAMC: NMT 10^3 bacteria/g; TYMC: NMT 10^2 CFU/g; absence of *S. aureus*, *P. aeruginosa*, *Salmonella* spp., and *E. coli*

Povidone	Disintegrant and suspending agent; dissolution aid; tablet binder; viscosity modifier, crystal growth inhibitor	Chemical—manufactured using the Reppe Process	None	None
Pregelatinized starch	Tablet and capsule diluent and disintegrant; tablet binder	Natural—manufactured using hot drums (approximately 62–72°C) where gelatinization and drying occurs	TAMC: NMT 10^3 CFU/g; TYMC: NMT 10^2 CFU/g; absence of *Salmonella* spp. and *E. coli*	TAMC: NMT 10^3 bacteria/g; TYMC: NMT 10^2 CFU/g; absence of *E. coli*
Shellac (lacca)	Coating agent	Natural—Shellac, also called "lac" is obtained from the resinous secretion of the female insect *Kerria lacca* (Kerr) Lindinger (*Laccifer lacca* Kerr); manufacture involves steam heat or solvent extraction with hot ethanol	None	None
Colloidal silicon dioxide	Adsorbent and anticaking agent; emulsion stabilizer; glidant; tablet disintegrant; thermal stabilizer; viscosity-increasing agent	Chemical—vapor hydrolysis of chlorosilanes at 1800°C using a hydrogen–oxygen flame	None	None
Sodium starch glycolate	Capsule and tablet disintegrant	Natural—chemical derivative of potato starch	Absence of *Salmonella* spp. and *E. coli*	Absence of *Salmonella* spp. and *E. coli*

Continued

TABLE 8.1 (Continued)
Common Excipients Used in U.S. Pharmaceutical Production

Excipient	Pharmaceutical Use	Source/Manufacturing Process	Microbial Specifications	
			USP31-NF26	Ph.Eur. (EP) 6.0 (2008)
Starch (corn)	Tablet and capsule diluent and disintegrant; tablet binder; glidant	Natural—extracted from plant sources; dried and milled	TAMC: NMT 10^3 CFU/g; TYMC: NMT 10^2 CFU/g; absence of *E. coli*; absence of *S. aureus* and *P. aeruginosa* if used in absorbable dusting powder formulation	TAMC: NMT 10^3 bacteria/g; TYMC: NMT 10^2 CFU/g; absence of *E. coli*
Stearic acid	Tablet and capsule lubricant; emulsifying and solubilizing agent	Chemical—hydrolysis of fat using high-temperature water in a high-pressure chamber	None	None
Sucrose	Sweetener; taste masking	Natural—obtained from sugarcane and sugar beet	None	None
Talc	Anticaking agent; tablet and capsule diluent and lubricant; glidant	Natural—naturally occurring hydropolysilicate mineral; purified and hydrated, magnesium silicate	**Topical administration:** TAMC: NMT 10^2 CFU/g; TYMC: NMT 50 CFU/g **Oral administration:** TAMC: NMT 10^3 CFU/g; TYMC: NMT 10^2 CFU/g	**Topical administration:** TAMC: NMT 10^2 bacteria/g; TYMC: NMT 10^2 CFU/g **Oral administration:** TAMC: NMT 10^3 bacteria/g; TYMC: NMT 10^2 CFU/g
Titanium dioxide	Pigment; coating and opacifier agent	Natural—occurs naturally as minerals rutile, anatase, and brookite	None	None

standardization for adventitious contamination of upstream biomanufacturing production samples is still needed. As far as downstream processing is concerned, the regulatory expectation is for companies to perform bioburden testing of in-process samples at various unit operations as a critical quality parameter indicator. Because biopharmaceutical production is typically carried out under bioburden-controlled rather than aseptic conditions, some level of bioburden is expected from in-process samples. However, these levels should remain stable or decrease downstream as an indication of bioburden control during the process. Typically, a level of not more than (NMT) 10 CFU per 10 mL is found acceptable for in-process downstream samples and a level of NMT 1 CFU per 10 mL is expected for the final nonsterile bulk drug API prior to final sterilization.

Nonsterile Finished Drug Products

Microbiological attributes for finished, nonsterile dosage forms are established based minimally on the following seven criteria:

a. Source or nature of raw materials
b. Water content
c. Product route of administration
d. Manufacturing process
e. Risk to target patient population
f. Product's ability to support or inhibit microbial growth
g. Potential for product degradation

Many compendial products have microbiological specifications provided in their respective monographs. In addition, the compendia provide guidance to drug manufacturers for assigning microbial specifications for nonsterile drug formulations and raw materials. Proposed microbial acceptance criteria are listed in the USP Chapter <1111>, *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Drug Substances for Pharmaceutical Use* [1]. Similar criteria are provided in the European Pharmacopoeia 6.0 [2], Section 5.1.4, *Microbiological Quality of Pharmaceutical Preparations* and in the Japanese Pharmacopeia XV [3], Chapter 12, *Microbial Attributes of Nonsterile Pharmaceutical Products,* where proposed specifications for herbal products are also included. Although recommendations for microbial attributes are provided in the compendia, it is still the manufacturing companies' responsibility to determine whether the proposed limits are suitable for their drug products and whether additional testing will be required based minimally on the seven criteria listed previously. In some cases, such as drug products targeted for patients with compromised or immature immune systems, microbiological specifications may need to be more stringent, especially because a wider variety of organisms may be potentially pathogenic to these patient populations.

USP Chapter <1111>

The USP Chapter <1111>, *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Drug Substances for*

Pharmaceutical Use, contains proposed microbial quality specifications based on the route of administration. This USP-harmonized (USP, EP, and JP) chapter was published in USP 30, and it has an official implementation date of May 01, 2009. This is a short chapter, not much longer than the original version, but this revision contains critical changes, which are summarized here:

1. *Tables with recommendations for microbial limits.* The chapter includes two tables with recommended acceptance criteria for microbiological quality of nonsterile dosage forms and nonsterile substances for pharmaceutical use. A similar table is provided in the revised and harmonized EP 6.0, Section 5.1.4, and in the JP XV, Chapter 12. In Table 8.2 of this book, a summary of these global compendial recommendations is presented.
2. *Clarification: Testing just to meet compendial microbial attributes is not enough*. The chapter clarifies that testing to meet compendial microbial limit requirements is not enough to satisfy the regulatory authorities. In this revised chapter, the USP clearly states that the given list of specified organisms for which acceptance criteria are set (refer to Table 8.2) is by no means *exhaustive*, and that it is the drug manufacturer's responsibility to perform additional testing as deemed appropriate. Such decisions must be made based on risk assessments, which should include evaluation of starting materials and the type of manufacturing process. In doing so, the firm can demonstrate compliance with the regulatory requirement specified in the 21 CFR 211.113 (a): "Appropriate written procedures, designed to prevent objectionable microorganisms in drug products not required to be sterile, shall be established and followed," and 21 CFR 211.165(b), "There shall be appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms." [4]
3. A result value twice the specification is acceptable. Interpretation of a passing bioburden test allows a result that is twice the value described in the product specification. For example, when an acceptance criterion for microbial quality is specified as 10 CFU, a maximum count of 20 CFU is acceptable; when an acceptance criterion for microbial quality is specified as 100 CFU, a maximum count of 200 CFU is acceptable; and so forth. This is a major positive change for pharmaceutical microbiologists in the United States and one that reflects the inherent variability in plate counting procedures. However, many companies are still hesitant to accept a product batch with a microbial count that exceeds the predetermined bioburden specification.
4. *Acknowledgment of limitations of current microbial recovery methods.* In this revised information chapter, the USP states that "none of the prescribed tests will allow valid enumeration of microorganisms at the level prescribed," and that "a validated method with a limit of detection as close as possible to the indicated acceptance criterion should be used." This statement clarifies and emphasizes the need for companies to validate compendial microbial methods to ensure reliability of test results.

TABLE 8.2
Global Microbial Quality Standards for Nonsterile Dosage Forms and Raw Materials

USP, EP, and JP	TAMC (CFU/g or CFU/mL)	TYMC (CFU/g or CFU/mL)	
Raw materials/substances for pharmaceutical use	10^3	10^2	
Route of Administration	**TAMC (CFU/g or CFU/mL)**	**TYMC (CFU/g or CFU/mL)**	**Specified Microorganisms**
Nonaqueous preparations for oral use	10^3	10^2	Absence of *E. coli* (per g or mL)
Aqueous preparations for oral use	10^2	10 50 (JP only)	Absence of *E. coli* (per g or mL)
Rectal use	10^3	10^2	Not specified
Oromucosal, gingival, cutaneous, nasal, or auricular use	10^2	10 50 (otic, nasal, or topical—JP only)	Absence of *S. aureus* (per g or mL); absence of *P. aeruginosa* (per g or mL)
Vaginal use	10^2	10 50 (JP only)	Absence of *P. aeruginosa* (per g or mL)—not in JP; absence of *S. aureus* (per g or mL); absence of *C. albicans* (per g or mL); absence of *E. coli* (per g or mL)—JP only
Transdermal patches	10^2	10 50 (JP only)	Absence of *S. aureus* (per patch including adhesive layer and backing); absence of *P. aeruginosa* (per patch including adhesive layer and backing)
Inhalation use (powder)	10^2	10	Absence of *S. aureus* (per g or mL); absence of *P. aeruginosa* (per g or mL); absence of bile-tolerant Gram-negative bacteria (per g or mL)—not in JP
Note: special requirements apply to liquid preparations for nebulization	20 (liquid, JP only)	50 (JP only) 20 (liquid, JP only)	

Continued

TABLE 8.2 (Continued)
Global Microbial Quality Standards for Nonsterile Dosage Forms and Raw Materials

EP and JP	TAMC (CFU/g or CFU/mL)	TYMC (CFU/g or CFU/mL)	Specified Microorganisms
Special provision for oral dosage forms that contain raw materials of natural origin with acceptable levels of TAMC exceeding 10^3 CFU per gram or per mL and for which treatment with antimicrobials is not possible (EP only).	10^4	10^2	Not more than 10^2 CFU of bile-tolerant Gram-negative bacteria (per g or mL); absence of *Salmonella* spp. (per 10 g or 10 mL); absence of *E. coli* (per g or mL); absence of *S. aureus* (per g or mL)
Special provision for solely herbal medicinal products to which boiling water is added before use	10^7	10^5 (EP) 10^4 (JP)	Not more than 10^2 CFU of *E. coli* (per g or mL); absence of *Salmonella* spp. per 10 g or 10 mL (JP only)
Special provision for solely herbal medicinal products to which boiling water is not added before use	10^5	10^4 (EP) 10^3 (JP)	Not more than 10^3 CFU of bile-tolerant Gram-negative bacteria (per g or mL); absence of *E. coli* (per g or mL); absence of *Salmonella* spp. (per 10 g or 10 mL)

Testing Frequency

It is a regulatory requirement, as specified in the 21 CFR Parts 210 and 211 Subpart F—Production and Process Controls, Section 211.100(a), that a company must have written procedures designed to ensure that the drug products manufactured have the identity, strength, quality, and purity they purport or are represented to possess [4]. Thus, all raw materials and nonsterile drug formulations that have a compendial requirement for microbial quality, to include the absence of specified microorganisms, must be tested for microbial content prior to approval for release. In addition, in-process testing should be performed so that the company is able to monitor the performance of the production process in its entirety. As far as frequency of testing is concerned, Section 211.110 (c) of the 21 CFR states that in-process materials should be tested "during the production process, e.g., at commencement or completion of significant phases or after storage for long periods" [4].

For biopharmaceutical products, Section 11.23 of the Q7 states that "appropriate microbiological tests should be conducted on each batch of intermediate and API where microbial quality is specified." [5] In general, both in-process and release materials are monitored for bioburden using a sampling plan that takes into account factors such as open versus closed operations, potential for equipment biofouling, and relation of the step to be sampled to a primary bioburden reduction step. Sampling should be performed in as many in-process steps as feasible, especially for processes in which no historical data are available. Column eluate pools, ultra filtration/diafiltration (UF/DF) pools, and samples from in-process hold steps are generally appropriate for evaluation of the microbial control of the process.

Stability Testing

As stated in the ICH document Q1A (R2), *Stability Testing of New Drug Substances and Products*, stability studies are carried out so that a drug manufacturer can ensure the quality of a drug substance or drug product under the influence of various environmental factors over time [6]. In addition, stability studies serve to provide data to justify retesting periods, shelf life, and recommended storage conditions for products. This document also states that testing performed during a stability program should include analyses for product attributes that are susceptible to change during storage and that are likely to influence the product's quality, safety, and/or efficacy; microbiological attribute is one of the recommended tests to be performed. Thus, bioburden testing is generally performed for stability samples because, depending on the product formulation, microbial contamination can increase over time and/or impact the chemical and physical attributes of the drug product under evaluation.

There are four global climatic zones as listed in Table 8.3, and these were established based on distinct, prevalent, annual climatic conditions. Stability protocols for drug products require samples to be held under given temperature and humidity conditions that simulate long-term, intermediate, and accelerated storage for a specific climatic zone. The choice of general test conditions defined in the ICH guideline Q1A (R2), and presented in Table 8.4, is based on climatic conditions in the United States, Europe, and Japan (climatic zones I and II). In this ICH document, it is stated

TABLE 8.3
Global Climatic Zones

Climatic Zone	Climate Definition	Storage Conditions
I	Temperate	21°C/45% relative humidity (RH)
II	Subtropical and Mediterranean	25°C/60% RH
III	Hot and dry	30°C/35% RH
IV	Hot and humid	30°C/70% RH

TABLE 8.4
ICH General Storage Conditions (General Case)

Study Protocol	Storage Conditions	Minimum Timeframe for Stability Data at Time of Regulatory Submission
Long term (applicant to decide which of the two to use)	1) 25 ± 2°C/60 ± 5% RH or 2) 30 ± 2°C/65 ± 5% RH	12 months
Intermediate (not needed if long-term storage option 2 is chosen)	30 ± 2°C/65 ± 5% RH	6 months
Accelerated	40 ± 2°C/75 ± 5% RH	6 months

that data generated under these conditions would be mutually acceptable by countries in other climatic zones provided the information is consistent with the given guideline. Further, the ICH steering committee has stated that the definition of storage conditions for drug products in climatic zones III and IV should be left to the respective regions and the World Health Organization (WHO).

In terms of frequency of testing during stability studies, the FDA recommends that for long-term study protocols, representative samples should be evaluated at a frequency sufficient to establish the stability profile of the drug product. In general, for a product with a proposed shelf life of at least 12 months, samples are tested every 3 months over the first year, every 6 months over the second year, and annually thereafter until the end of the proposed shelf life. For accelerated stability studies, the FDA recommends a minimum of three time points; for example, for a 6-month study, stability samples should be tested at time points 0, 3, and 6 months. For an intermediate storage condition protocol, typically a minimum of four time points are required, including the initial and final time points.

For bioburden testing during a stability program, most companies choose the following time points to be evaluated: 0 (initial), 6, 12, 18, 24, 36, 48, and 60 months. Testing at a higher frequency is usually not practical for microbial analysis and often a non–value-added activity. Factors that are often taken into account when designing a bioburden testing program in support of a stability study for nonsterile drugs and raw materials include the drug product formulation (chemistry and water activity) and type of manufacturing process. Based on the chemistry of some formulations

(e.g., extreme pH, oxidation potential, alcohol content, and low water activity), many types of microorganisms would not survive or thrive during storage. The manufacturing process itself can be a hostile environment to microorganisms that may be present in raw materials. Therefore, using a risk-based approach, scientific rationale, and/or review of historical data, companies can justify their choices for the microbiological testing program.

WATER ACTIVITY

Water associated with substances is either free or bound. The terms *water activity* (a_w) or *equilibrium-relative humidity* (ERH) is a measure of the unbound (free) water in a material that is available for chemical and biological reactions. Water activity is defined as the ratio of water vapor pressure of a substance (p) to the vapor pressure of pure water (p_o) at the same temperature (i.e., $a_w = p/p_o$). Therefore, ERH can be defined as water activity expressed as a percentage (ERH = $a_w \times 100$). Water activity is not the same as moisture content, although materials that have high moisture are most likely to have greater water activity as compared to dry ones.

Water activity is a product characteristic that has been widely used in food industry to control microbial contamination. The concept of a_w has now been introduced in the pharmaceutical industry as a valuable tool not only during drug product development but also as a possible alternative to microbial limit testing. In addition to microbial concerns, water activity can also impact protein stability due to aggregation and conformational changes that can take place above critical water activity levels. Thus, knowledge of the water activity of pharmaceutical/biopharmaceutical formulations is key to ensure chemical and microbial stability of the product.

Among the many environmental factors that influence microbial growth, the availability of water for biological processes is probably the most important, because microorganisms have a limiting water activity level below which they cannot maintain viability or proliferate. The scale used to represent water activity extends from zero (extremely dry) to 1.0 (pure water). Most bacteria require a water activity level typically greater than 0.93 for proliferation. Table 8.5 contains a list of selected representative microorganisms and their minimum water activity requirements for survival. Osmophilic organisms (those that live in environments high in sugar) and halophilic organisms (those that require sodium chloride for growth) are somewhat more tolerant to lower water activity levels. However, once the a_w in a material drops below 0.6, the chance of microbial growth, even for xerophilic microbes (i.e., organisms adapted to growth in very dry environments), is greatly diminished, as illustrated in Figure 8.1.

The application of water activity in the pharmaceutical and biopharmaceutical industries was first addressed in the USP Stimuli to the Revision Process article, "The Application of Water Activity Measurement to the Microbiological Attributes Testing of Nonsterile Over-the-Counter Drug Products," by Friedel and Cundell [7]. Published as a draft chapter in Pharmacopeial Forum (PF) 28(6) 2009–2013 [Nov.–Dec. 2002], the new USP Chapter <1112>, *Application of Water Activity Determination to Nonsterile Pharmaceutical Products,* was finally made official on August 1, 2006, in USP 29, Supplement 2. In this informational chapter, the USP acknowl-

TABLE 8.5
Water Activity Levels Required to Support Microbial Growth

Microorganism	Type	Water Activity (a_w) (Typical Values)
Pseudomonas aeruginosa	Bacteria (Gram-negative rod)	0.97
Bacillus cereus	Bacteria (Gram-positive, spore forming)	0.95
Clostridium botulinum	Bacteria (Gram-positive rod, spore forming, anaerobe)	0.95
Clostridium perfringens	Bacteria (Gram-positive rod, spore forming, anaerobe)	0.95
Escherichia coli	Bacteria (Gram-negative rod)	0.95
Salmonella spp.	Bacteria (Gram-negative rod)	0.95
Lactobacillus viridescens	Bacteria (Gram-positive rod; facultative anaerobe)	0.95
Enterobacter aerogenes	Bacteria (Gram-negative rod)	0.94
Rhyzopus nigricans	Filamentous fungus	0.93
Micrococcus lysodekticus	Bacteria (Gram-positive coccus)	0.93
Rhodotorula mucilaginosa	Yeast	0.92
Bacillus subtilis	Bacteria (Gram-positive, spore forming)	0.90
Saccharomyces cerevisiae	Yeast	0.90
Staphylococcus aureus	Bacteria (Gram-positive coccus)	0.86
Paecilomyces variotti	Filamentous fungus	0.84
Penicillium chrysogenum	Filamentous fungus	0.83
Aspergillus fumigatus	Filamentous fungus	0.82
Aspergillus niger	Filamentous fungus	0.77
Halobacterium halobium	Archaea (halophilic archaean)	0.75
Zygosaccharomyces rouxii	Osmophilic yeast	0.62
Xeromyces bisporus	Xerophilic filamentous fungus	0.61
No microbial proliferation below this level		0.50

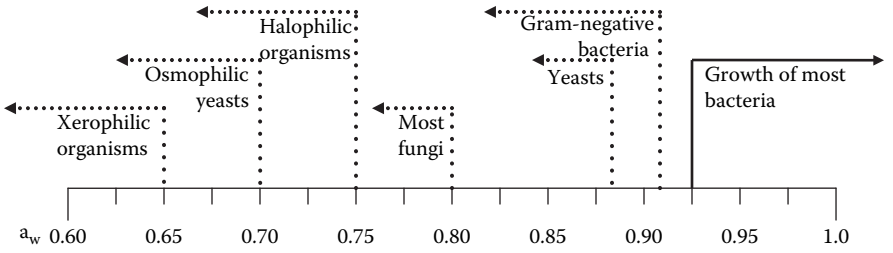

FIGURE 8.1 Inhibition of microbial growth based on water activity.

edges that water activity determination can have many beneficial applications to the pharmaceutical industry, including

- Optimizing product formulations to improve antimicrobial effectiveness of preservative systems
- Reducing susceptibility of drug product formulations to microbial contamination
- Providing companies with a tool to justify reducing the frequency of bioburden/microbial limit testing and screening for objectionable microorganisms for product release and stability testing

Measuring Water Activity

Water activity is determined by indirect measurements using primarily two types of methods: one measures changes in resistance (capacitance), and the other the dew point.

The Chilled-Mirror/Dew Point Method

This is the primary method approved by the AOAC International [8] and one that is referenced in the USP Chapter <1112>. This method measures the dew point temperature, that is, the temperature when the air becomes saturated in equilibrium with water. Because air may be cooled without changes in water content until the air saturates, the dew point temperature is determined by measuring the temperature of a chilled mirror when condensation begins. The water activity of the sample is then calculated as the ratio of the saturation vapor pressure at the dew point temperature to the saturation vapor pressure at the product temperature.

The chilled-mirror test is carried out by placing the sample in a sealed container against a sensor block that holds a dew point sensor (measures the dew point temperature of the air), an infrared thermometer (measures sample temperature), and a fan for circulating the air to reduce the vapor equilibrium time and control the boundary layer conductance of the mirror surface. A beam of infrared light directed onto the mirror is reflected back to a device that detects changes in reflectance when the first condensation occurs on the mirror. Measurement of the headspace in the container when the water activity is in equilibrium with the relative humidity in the air provides the water activity of the material. Figure 8.2 shows the AquaLab water activity meter, series 3TE (Decagon Devices, Inc., Pullman, WA), which features an internal temperature control to avoid erroneous results due to fluctuations in ambient temperature in the testing environment.

Capacity Sensors

Water activity can be determined using instruments that measure the ERH of the air surrounding the material being tested. This type of method is based on the principle that at equilibrium the relative humidity of the air in a closed chamber is equal to the water activity in the material held in that environment. Because water activity is temperature dependent, the measured ERH is equal to the water activity level in the sample only when the temperatures of the sample and the sensor are identical.

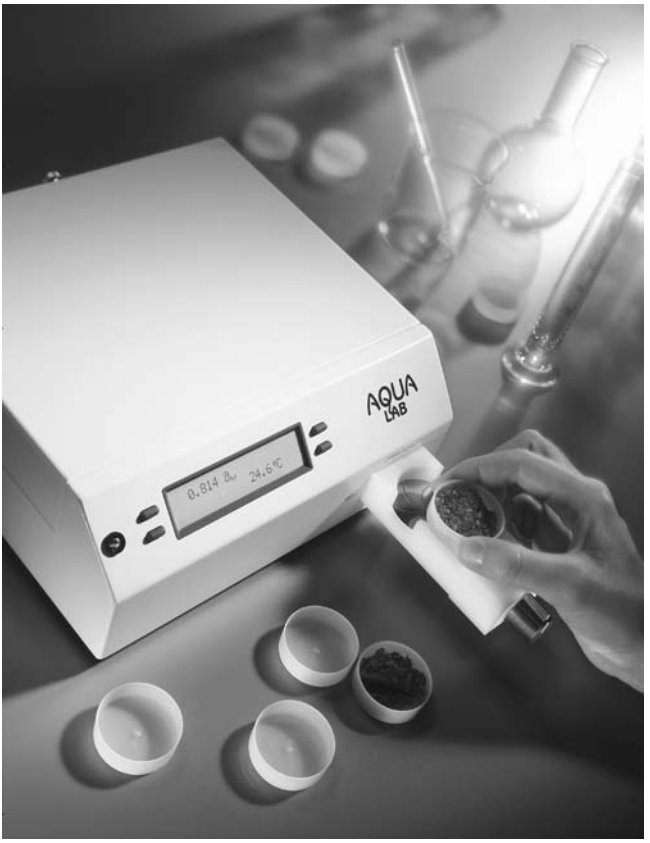

FIGURE 8.2 AquaLab water activity meter, series 3TE. (Decagon Devices Inc., Pullman, WA. With permission.)

However, using this type of method usually takes longer because capacity sensors take about 30 to 90 min to reach equilibrium of temperature and vapor.

When comparing these two types of instruments, the chilled-mirror method offers advantages in terms of accuracy, speed of test (results are usually available within 5 min), ease of use, and above all, being a test recommended in the USP. Therefore, it is not surprising that most testing laboratories have adopted this method for determination of water activity, even though in terms of cost its apparatus is usually more expensive compared to other devices used for the same application.

Pharmaceutical Applications for Water Activity

As stated in the USP Chapter <1112> and discussed in this chapter, knowledge of the water activity of pharmaceutical products is essential to produce nonsterile solid dosage forms and biopharmaceuticals of good chemical and microbial quality as well as optimum shelf-life properties. In addition, water activity can be used as a tool to reduce the frequency or eliminate the need for microbial limit testing for certain products. For example, nonaqueous products such as tablets and powder-filled

TABLE 8.6
USP-Proposed Microbial Limit Testing Strategies

Product Type	Typical a_w	Potential Contaminants	Testing Recommended in USP <1111>	Testing Recommended in USP<1112> Based on a_w
Nasal liquid inhalant	0.99	Gram-negative rods	TAMC and TYMC; absence of *S. aureus* and *P. aeruginosa*	TAMC and TYMC; absence of *S. aureus* and *P. aeruginosa*
Topical cream	0.97	Gram-positive bacteria	TAMC and TYMC; absence of *S. aureus* and *P. aeruginosa*	TAMC and TYMC; absence of *S. aureus* and *P. aeruginosa*
Oral liquid	0.90	Gram-positive bacteria and fungi	TAMC and TYMC; absence of *E. coli*	TAMC and TYMC
Oral suspension	0.87	Fungi	TAMC and TYMC; absence of *E. coli*	Reduced testing
Topical ointment	0.55	None	TAMC and TYMC; absence of *S. aureus* and *P. aeruginosa*	Reduced testing
Compressed tablets	0.36	None	TAMC and TYMC; absence of *E. coli*	Reduced testing
Vaginal suppositories	0.30	None	TAMC and TYMC; absence of *S. aureus, P. aeruginosa,* and *C. albicans*	Reduced testing
Liquid-filled oral capsules	0.30	None	TAMC and TYMC; absence of *E. coli*	Reduced testing

capsules that are manufactured with materials that meet bioburden specification are good candidates for reduced bioburden testing in support of product release and/or during stability studies. A summary of the USP-recommended testing strategies for microbial limit testing when the water activity in the product is known is provided in Table 8.6. As noted in the USP, this information is intended to provide guidance only. It is a regulatory expectation that manufacturers should carefully assess their testing strategies based on the types of products manufactured and intended patient population, because more resistant organisms, such as spore-forming bacteria and filamentous fungi, may still survive in a material with low water activity.

INTERNATIONAL HARMONIZATION

Global trade in drug products and raw materials has prompted the pharmaceutical industry to work toward international harmonization of standards, specifications, manufacturing processes, and methodologies. However, geographic expansion comes with both rewards and risks: rewards are seen in terms of speed to market by using contract manufacturing companies and raw material suppliers in countries such as India and China that have the know-how and skilled work force to carry

out the work in a very economical fashion; risks are many, and mostly in terms of the providers' ability to consistently meet internationally recognized high-quality and regulatory standards for pharmaceutical production. For raw materials, regulations regarding sampling and testing vary globally, creating even greater business and compliance challenges. Nothing can stop pharmaceutical operations more rapidly than raw materials that do not meet quality standards. Indeed, regulatory issues can become a stumbling block in manufacturing operations when there is a lack of agreement between local government expectations and local business requirements. Although emerging markets are attempting to model their operations and quality systems after the ones seen in the European Union and the United States, there are still vast differences that must not be overlooked. Therefore, in order to be successful, companies should acquire an in-depth knowledge of the local regulations and potential risks that could negatively impact business operations. It is critical for pharmaceutical firms that are expanding into developing markets or purchasing raw materials from developing countries to find out whether suppliers in those areas are ISO 9001 certified and to perform vendor quality audits. Holding an ISO certification is essential as it ensures that the company has been evaluated by an independent and qualified third party and found to have a quality system that meets internationally recognized production quality standards.

One of the most difficult hurdles to overcome in global harmonization has been the standardization of test methods, mainly due to factors such as principles of validation and availability of reagents, equipment, and supplies. However, during recent years, the exchange of scientific knowledge among regulatory authorities worldwide has made it easier to discuss harmonization of test methods in an attempt to reach consensus. Indeed, the goal of harmonization is not to reach a unanimous decision but, rather, as the meaning of the word indicates, to reach *harmony.* In the three pharmacopoeias discussed in this book (USP, EP, and JP) and which have been the cornerstones for harmonization initiatives, provisions have been made to allow for alternate media, reagents, challenge organisms, and even methodologies to test the microbial attributes of pharmaceutical products and ingredients.

Looking Ahead …

During the next decades, the author believes that there will be a continued effort toward implementation of faster, more accurate, and more reliable procedures for microbiological testing. Perhaps automation and the use of new and alternate technologies will finally take center stage in QC microbiology laboratories and pharmaceutical microbiologists, at last, will enjoy the technological advances of the 21st century!

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9 Rapid Testing and Alternative Methods in Microbiology

Microbial testing performed in support of pharmaceutical and biopharmaceutical production falls into three main categories: detection (qualitative), enumeration (quantitative), and characterization/identification. Traditional microbiological methods listed in the compendia and discussed in various chapters of this book use conventional growth-based techniques, which are labor intensive and time consuming. In general, such tests require days of incubation for microbial contamination to be detected, and therefore management seldom is able to take proactive corrective measures. In addition, microbial growth is limited by the growth medium used and incubation conditions, thus impacting testing sensitivity, accuracy, and reproducibility.

For more than 20 years various technology platforms for rapid microbiological methods (RMM) have been developed, and many have been readily adopted by the food industry and clinical microbiology laboratories. Their use would certainly offer drug companies faster test turnaround times to accommodate the aggressive deadlines for manufacturing processes and product release. Some rapid methods also offer the possibility for real-time microbial analyses, enabling management to respond to microbial contamination events in a more timely fashion, and can provide cost savings and higher efficiencies in quality control testing laboratories. In fact, RMM was listed on a recent article in the PDA Journal of Pharmaceutical Science and Technology as one of the top ten hot topics for the pharmaceutical industry [1].

Despite the many proved business and quality benefits and the fact that the FDA's initiative to promote the use of process analytical technology (PAT) includes rapid microbial methods [2], pharmaceutical and biopharmaceutical industries have been somewhat slow to embrace alternative microbial methodologies for reasons discussed later in this chapter.

Rapid methods is a dynamic field in applied microbiology and one that has gained increased attention nationally and internationally over time. This topic has been extensively addressed at conferences and in published documents

around the world. More recently, the use of alternative methods for control of the microbiological quality of pharmaceutical products and materials used in pharmaceutical production has been addressed by the compendia in an attempt to facilitate implementation of these technologies by pharmaceutical companies. In the EP 6.0, Chapter 5.1.6, *Alternative Methods for Control of Microbiological Quality*, an entire section is dedicated to method validation, and a detailed validation protocol for bioluminescence is included.

In this chapter, the author presents some of the rapid method technologies under evaluation or in use by pharmaceutical microbiologists and the current status of implementation of alternative microbial methods. The subject of method validation is addressed in Chapter 7 of this book.

RAPID METHOD TECHNOLOGY PLATFORMS

Rapid methods and automation deal with the study and development of improved techniques for isolating, detecting, characterizing, and enumerating microorganisms. RMM include automated compendial tests as well as alternative technologies. Automated compendial methods use the same technology principles as traditional microbiological tests; the difference is that the procedure used for testing has features that allow automation. For example, an automated colony counter enumerates microbial colonies that have grown on solid medium using digital imaging, thus expediting the counting process. Alternative technologies, which for the most part are also automated, enumerate or detect microorganisms using distinct methodologies. Therefore, in many cases, measurements obtained via alternate methods may not match those obtained using traditional testing methods. For example, results for a total viable count performed using a traditional plate count are reported as number of CFU, whereas results based on ATP bioluminescence are reported as number of relative light units (RLU), which may not correlate to the number of CFU isolated.

Microbial rapid methods evolved from simple miniaturization of test kits in the 1960s and 1970s to the use of alternate technologies, such as molecular biology techniques, that were developed in the 1980s and 1990s. In the 21st century, microbiologists have witnessed the development of microbial methods using computer chip technology (microchips) and microarray systems. Along with the journey toward development of alternate microbial methods, scientists coupled technologies with automated instrumentation that would provide for more efficient testing.

The Encyclopedia of Rapid Microbiological Methods [3], the *Handbook on Rapid Methods and Automation in Microbiology* [4], and the 8th edition of the *Manual of Clinical Microbiology* [5] provide comprehensive information on RMM, which can be grouped into four main technology platforms:

1. *Growth-based methods.* Testing performed measures biochemical reactions and the organism's physiological changes as a result of microbial growth under specified conditions. Instrumentation used in growth-based rapid

methods includes equipment that can measure electrical impedance/conductivity of the test solution, biochemical reactions (e.g., carbon assimilation, enzymatic reactions, and CO_2 generation), or ATP bioluminescence.
2. *Artifact-based methods.* Testing involves analysis of components from microbial cells. Examples of artifact-based technologies include fatty acid analysis using gas chromatography, ELISA, and MALDI-TOF mass spectrometry of cell components such as nucleic acids and proteins. Bacterial endotoxin testing is also an artifact-based test. However, because this test is outside the scope of this book, it will not be discussed in this chapter.
3. *Nucleic-acid-based methods.* Testing performed involves amplification of microbial DNA using polymerase chain reaction (PCR) protocols and riboprinting techniques (automated Southern Blotting). These methods are used for the purpose of microbial identification and interstrain differentiation, the latter being a very useful technique during investigations of microbial contaminations.
4. *Viability-based methods.* Testing performed involves the use of viability stains or biological markers that are capable of detecting and enumerating microorganisms without the need for incubation to increase cell density. Examples of viability-based technologies include fluorescent labeling methods, such as flow fluorescence cytometry; immunofluorescence (based on the fluorescent labeling of cells using an antiserum raised in rabbits); and fluorescent nucleic acid stains used as a viability marker along with propidium iodide as a membrane-compromised cell marker.

According to recent publications, such as the report on New Technologies Forum 6, Rapid Methods in Microbiology, held at the Royal Pharmaceutical Society in February, 2003 [6], the main systems either in use today or being evaluated by the pharmaceutical/biopharmaceutical industry are based on technologies that include automated biochemical reactions (metabolic fingerprinting), fluorescent labeling assays, impedance/conductivity, gas consumption or production, ELISA, PCR, fatty acid analysis using gas chromatography, ATP bioluminescence, riboprinting, and analysis of biomolecules using mass spectrometry. An overview of these technologies, along with a discussion on biosensors and microarrays, follows.

Impedance/Conductance Technology

Impedance can be defined as a measure of the overall opposition of a circuit to an electric current; in other words, how much the circuit impedes the flow of current. Although similar to resistance in concept, impedance is viewed as a more complex measure of electrical flow through a medium because it takes into consideration the effects of capacitance (amount of electrical charge stored for a given electric potential) and inductance (the ratio of the magnetic flux produced when an electric current flows, to the electric current). Both capacitance and inductance vary with the frequency of the electrical current passing through a circuit. Therefore, impedance varies with frequency, whereas resistance is constant regardless of the frequency. *Conductance* is the reciprocal of electrical impedance, that is, it is a measure of how

easily electricity flows through a medium. Impedance is measured in Ohms (Ω), and conductance is measured in Siemens per cm (Scm^{-1}).

Although first described almost 100 years ago, impedance/conductance microbiology did not evolve until the mid-1980s. This technology is based on the fact that as microbes grow, they metabolize large, weakly charged molecules (polysaccharides, proteins, and fats) to produce small, highly charged molecules (organic acids, fatty acids, and amino acids), resulting in a change in electrical conductivity and resistance of the growth medium, which can be detected using two electrodes. In general, when the microbial population in a culture medium reaches a certain number, for example, 10^5 per mL medium [7], changes in these parameters can be detected. The time it takes for the equipment to detect changes is inversely proportional to the number of organisms in the medium—the smaller the initial microbial count, the longer the detection time.

Impedance/conductance technology has potential use for testing efficacy of antimicrobial products and for detection of microbial contamination in samples. The **Bactometer®** (bioMérieux, Inc., www.biomerieux-usa.com), based on impedance technology, has been successfully used for qualitative and quantitative microbial testing of foods, cosmetics, water, and pharmaceutical products for many years. However, the vendor is no longer making this system and plans to phase it out soon. Other systems available on the market include **BacTrac** (Sy-Lab, www.sylab.com), **Rapid Automated Bacterial Impedance Technique** (**RABIT**; Microbiology International, www.microbiology-intl.com), and the **Malthus Microbial Detection System** (Malthus Diagnostics, North Ridgeville, Ohio) which are based on conductance measurements and have AOAC International-approved testing protocol for detection of *Salmonella* spp. Automated systems based on impedance/conductance technology offer a 1–4 d advantage in test turnaround time over traditional microbial plating methods and eliminate the need for serial dilutions. In a study that used the Malthus instrument for detection of biofilms of thermophilic bacteria on stainless steel surfaces, the researchers concluded that there were additional advantages to the use of the conductance/impedance technology; the results generated were faster and more accurate for estimation of the number of surface biofilm cells when compared to traditional acridine orange epifluorescence and swab recovery methods [8].

Gas Consumption or Generation

Microorganisms that actively metabolize in growth media consume certain gases (e.g., aerobes consume oxygen), which lead to the production of metabolites (e.g., production of carbon dioxide). Certain types of rapid method equipment measure changes in the gaseous head-space composition of a closed culture vessel using pressure transducers. Other systems employ colorimetric detection of carbon dioxide (CO_2). This technology is very effective for detection of slow-growing microorganisms.

The **BacT/ALERT® 3D** (bioMérieux, Inc., www.biomerieux-usa.com), shown in Figure 9.1, is an automated microbial detection system based on bioMérieux's patented colorimetric technology. This sensor-and-detection technology detects microorganisms by tracking CO_2 production. As microorganisms grow and multiply in the media, they generate CO_2, and as the concentration of this gas increases, the sensor

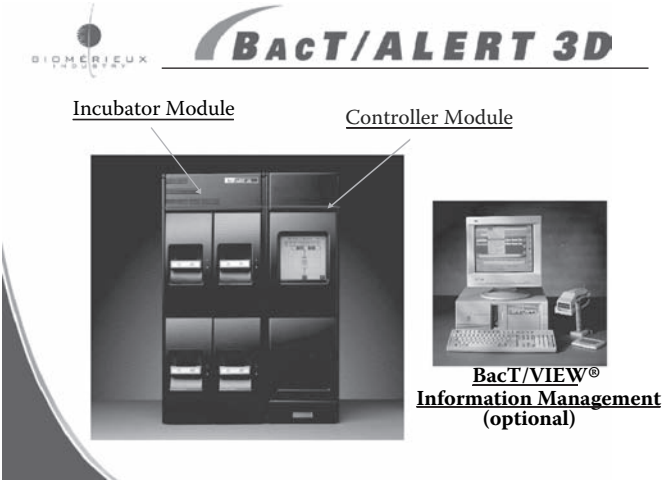


FIGURE 9.1 The BacT/ALERT® 3D microbial detection system based on patented colorimetric sensor-and-detection technology for tracking CO_2 production. (Courtesy of bioMérieux, Inc., www.biomerieux-usa.com. With permission.)

in the bottle turns yellow. The BacT/ALERT 3D measures reflected light to monitor and detect color changes in the sensor that are permanent and visible to the naked eye. Using algorithms, the data generated are analyzed to determine the presence of microbial growth (positivity). The BacT/ALERT 3D is able to detect a wide range of organisms, with greater than 95% recovery within 24 h and greater than 98% within 72 h.

ATP Bioluminescence

Bioluminescence can be defined as a high-efficiency generation of light by biological systems as a result of chemiluminescent reactions that take place in a protein environment. Since the mid-1940s, when William D. McElroy discovered bioluminescence properties while studying fireflies [9], this science has been developed for different applications to include rapid methods in microbiology.

Bioluminescence is dependent on adenosine 5′-triphosphate (ATP), which is present in all living organisms and is used as a marker of cell viability. ATP is a multifunctional nucleotide produced as an energy source during processes such as photosynthesis and cellular respirations, and consumed (ATP to ADP) by many enzymes during numerous anabolic processes that require energy. Under certain conditions, for example, when ATP released from microorganisms combines with the enzyme luciferase from the firefly, it is converted to a photon having a yellow-green color; here, the luciferase hydrolyzes the ATP to adenosine monophosphate (AMP), and the stored energy is released as light, as shown in the mechanism of reaction described as follows:

$$\text{ATP} + \text{D-Luciferin} + \text{O}_2 \xrightarrow[\text{Mg}^{+2}]{\text{Luciferase}} \text{AMP} + \text{Oxyluciferin} + \text{CO}_2 + \text{PPi} + \text{Light}$$

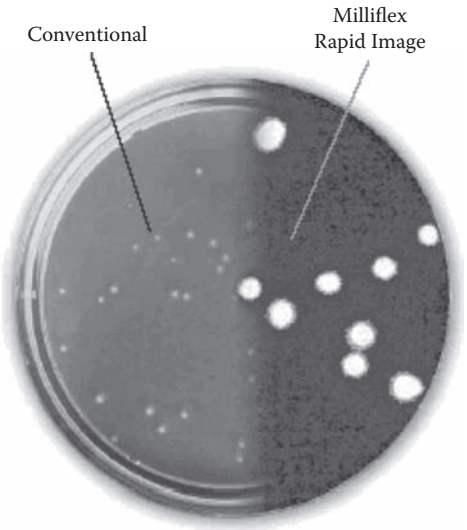


FIGURE 9.2 Conventional (left) versus ATP-Bioluminescence rapid image analysis (right) using the Milliflex® Rapid Microbiology Detection System. (Photo courtesy of Millipore Corporation, www.millipore.com. With permission.)

Automated instruments equipped with photometers able to detect visible light (approximately 390–620 nm) are used to measure bioluminescence in many industrial applications; this also involves the detection and quantitation of microbial contamination. These instruments are able to convert light into an electronic signal or pulse, and results are reported in terms of RLU. Studies can be performed to correlate RLU with CFU so that calibration graphs are generated. Such studies, which must be product specific, are carried out by inoculating the product/test material with known levels of organisms, such as 10, 100, 500, and 1000 CFU, processing the samples according to the testing protocols, and observing the resulting RLU for a possible correlation with the known inoculum level. Different types of organisms must be used in such qualification studies because there are differences in the level of ATP among the various types of microbes. Although a correlation between RLU and CFU can be established, an ATP-based assay is still not quantitative; all one can say is that there is less than or more than a certain number of CFU present in the sample. Alternate plate-counting methods are still needed for accurate bioburden determination.

For microbial quantitation and detection, a sample enrichment step is required to ensure that there is sufficient ATP present for detection. Incubation times vary from 6 to 18 h. The letheen medium is commonly used because of its low background levels of ATP and its good neutralizing capabilities. Other media possessing low background levels of ATP are sabouraud dextrose broth and nutrient broth. After incubation, an ATP-releasing agent is added to the sample, followed by addition of a reagent to react with the ATP and produce light, which is then measured by a luminometer (see Figure 9.2).

There are some concerns with this technology as far as test accuracy, reproducibility, and sensitivity are concerned, as listed here:

1. The technique detects nonmicrobial ATP. A high level of nonmicrobial ATP in a product will raise the baseline of microbial ATP resulting in a less sensitive method.
2. Different types of microbes have different amounts of ATP per cell. For example, a yeast cell may have 100 times more ATP compared to a bacterial cell. In addition, for the same organism, ATP levels per cell vary depending on the growth stage.
3. Results are impacted by the effect on background ATP caused by other types of biomass, such as biological fluid, and turbidity and color of the sample.
4. Real-time microbial detection is possible only in cases where high levels of microbial contamination is present (e.g., hygiene monitoring) because this technology requires some incubation period to increase cell mass for samples with expected low-level bioburden.

Evaluations to assess some of these concerns should be conducted during method validation studies. These studies are performed to determine the required sample preparation incubation time, to ensure that there is no interference in the test from nonmicrobial ATP, and to ensure that the product/material is suitable for microbial evaluation using the ATP bioluminescence technology. Currently, due to such known interferences, the use of systems based on ATP bioluminescence is limited to real-time hygiene and environmental monitoring procedures, and to the testing of products that do not interfere with the detection of microbial ATP. The application for hygiene and environmental monitoring is quite good because this technique is able to show the level of cleanliness of equipment or manufacturing surfaces. Some systems available on the market allow for instantaneous feedback on the hygiene status of the production environment. By measuring the total ATP on the surfaces, the instrument provides a measure of overall cleanliness; however, it is not correlated to certain levels of microorganisms. Usually, the bioluminescence test is a "pass" or "fail" test. If, for example, the result obtained is twice as high as the blank control, the product or surface fails the test, indicating that ATP is present. Additional testing would be performed for estimating the microbial population. On the other hand, studies have shown that when a suitable medium is used, and there is no product interference, this technique is able to detect very low levels of microorganisms (in the range of 5–10 CFU).

Despite some of the limitations of this technology as a quantitative microbial detection method, ATP bioluminescence has a great potential for pharmaceutical applications as an endpoint microbial detection system. This technology can be used to expedite detection of microbial contamination on equipment surfaces and in products suitable for this technology. Rapid systems with filtration capabilities have had a revolutionizing effect on the testing of water samples and product sterility.

Some of the commercial systems available on the market include the **Milliflex® Rapid Microbiology Detection System** (Millipore Corporation, www.millipore.com) (shown in Figure 9.3), the **Pallcheck™ Rapid Microbiology System** (Pall Corporation, www.pall.com), and the **Celsis Advance™** (Celsis International plc, www.celsis.com) (shown in Figure 9.4).

FIGURE 9.3 The Milliflex® Rapid Microbiology Detection System. (Photo courtesy of Millipore Corporation, www.millipore.com. With permission.)

FIGURE 9.4 The Celsis Advance™ System. (Photo courtesy of Celsis International plc, www.celsis.com. With permission.)

The Celsis ATP Bioluminescence Systems

The Celsis Advance has been designed for in-process testing of pharmaceutical products. This system is 21 CFR Part 11 compliant, and it features dedicated software for industrial microbiology laboratories with capability to accommodate in-house testing protocols. There is no need for onboard sample incubation, and the system has a large sample capacity (up to 164 samples). Celsis also manufacturers the **AKuScreen™,** the next generation in ATP bioluminescence technology, which is based on the Adenylate Kinase assay technology exclusively licensed to Celsis by

the British Defense Science and Technology Laboratory (Dstl). Using this technology, method sensitivity is increased as microbial detection is accomplished via two enzyme-catalyzed reactions: during the first reaction, ATP is produced from ADP in the presence of the enzyme adenylate kinase. The second reaction is the typical catalyzation of ATP into light in the presence of the enzyme luciferase. Using this latest technology, which effectively generates and amplifies ATP in the sample, detection occurs sooner than with traditional ATP bioluminescence methods—in as few as 18 h for most organisms; mold detection occurs in about 24 h.

Automated Biochemical Assays

Some of the first semiautomated biochemical methods used for identification of microorganisms were developed in the mid-1970s. Test kits such as the API® system (bioMérieux, Inc., www.biomerieux-usa.com) standardized the preparation of biochemical substrates and culture of inocula; however, final interpretation was still in the hands of the microbiologist.

In the 1980s, fully automated systems became available with the on-line matching of biochemical profiles of unknown organisms with a database having biochemical profiles of known organisms. Automated microbial identification instruments are limited by their databases because they can correctly identify only those organisms for which representative or reference strains have been entered into standard or customized libraries. Otherwise, the result will indicate "no match" or "unindentified organism." In certain cases, the system will give the most probable match based on close species similarities and recommend additional testing for confirmation of identification.

The VITEK® System

One of the best known systems on the market for microbial identification is the **VITEK® 2 Compact**, shown in Figure 9.5. The first generation of VITEK® Systems was developed in the late 1960s in a joint venture between McDonnell Douglas and NASA. Today, it is marketed by bioMérieux, Inc. (www.biomerieux-usa.com), a medical device manufacturer that is ISO 9001 certified and monitored by a variety of agencies such as the FDA.

BioMérieux launched the 2nd generation instrumentation in 2003. The VITEK® 2 Compact is a fully automated identification and susceptibility system based on microbial utilization of substrates and enzymatic reactions, and fluorescent technology. The system consists of the following components:

- Computer
- Data terminal/keyboard/mouse
- Printer
- Disposable test Cards
- Filler/sealer module
- DensiChek
- Reader/incubator module
- Software

FIGURE 9.5 The VITEK® 2 Compact Microbial Identification System. (Courtesy of bioMérieux, Inc., www.biomerieux-usa.com. With permission.)

The disposable test kits are miniaturized (10 × 6 × 0.5 cm) plastic cards containing double the reagent test wells of the previous system. Each consists of 64 wells with different well combinations utilized to identify various types of microorganisms. This, coupled with three wavelengths for reading, allows for inclusion of more organisms in the database than with the first generation technology. Card types contain different dehydrated biochemical substrates configured for the specified use. Therefore, in order to choose an appropriate test kit, it is necessary to isolate a pure culture of the unknown for Gram staining. No other external tests are needed with the VITEK® 2 Compact. The previous system required oxidase and catalase reactions to determine the correct Card for use. After primary isolation, a suspension of the unknown is prepared in a tube of sterile saline solution and verified with the DensiChek densitometer. The inoculated tube is then placed into a rack (the cassette), and the sample identification number is entered into the Smart Carrier via barcode or keypad and electronically linked to the supplied barcode on each test Card. The use of external marks, required for the VITEK®, is no longer required for the VITEK® 2 Compact. Cards have barcodes that provide all the necessary information for each test. After loading the test Card and inoculum tube into the system, the sample is automatically drawn into the test wells via a vacuum source. After inoculation, the Card is sealed and incubated. All processing steps are completely automated and standardized. Biochemical reactions are monitored initially and at frequent intervals during the incubation period. For example, the optical system for the VITEK® 2 Compact reads all 64 wells every 15 min utilizing three wavelengths, in a kinetic-type data collection. Data analysis and interpretation are performed automatically using a comparison of the biochemical profile of the unknown organism with the standardized biochemical profiles contained in the microbial identification database. A final report is printed automatically for each test kit at the end of its cycle (Figure 9.6). The identification of the organism is printed along with the different biochemical reaction results observed for each well (negative/positive) according to which a Bionumber is assigned. The Bionumber is useful for trending data and for evaluation of the microorganisms' similarities.

Industry System

bioMerieux Customer: 123456789 Laboratory Report Printed Feb 3, 2006 11:16 CST
System #: 987654321 Printed by: LabSuper
Report Version: 1 of 3

Isolate Group: 1111-1

Bionumber:0405611540566650
Selected Organism: Escherichia coli

Comments:	

Identification Information	Card: GN	Lot Number: 241010000	Expires: Mar 24, 2007 12:00 CST
	Completed: Sep 28, 2005 15:52 CDT	Status: Final	Analysis Time: 5.75 hours
Selected Organism	99% Probability	Escherichia coli	
	Bionumber: 0405611540566650		Confidence: Excellent identification

SRF Organism	
Analysis Organisms and Tests to Separate:	
Analysis Messages:	
Contraindicating Typical Biopattern(s)	

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	lMLTa	+	62	ELLM	-	64	lLATa	-			

VITEK 2 Compact Version: 01.02 Therapeutic Interpretation Guideline:
MIC Interpretation Guideline: AES Parameter Last Modified:
AES Parameter Set Name:

FIGURE 9.6 Sample report generated by the VITEK® 2 Compact system. (Courtesy of bioMérieux, Inc., www.biomerieux-usa.com. With permission.)

The VITEK® 2 Compact microbial identification system has extensive clinical databases but was designed to enhance the capabilities of its environmental/industrial libraries. The entire database is comprised of hundreds of microbial species, and these are routinely updated to accommodate other/new strains and latest microbial nomenclature. The software also allows industrial or nonclinical users to create customized databases or supplemental react file with identification results for environmental and product isolates which may not appear in the database. The optional Observa Data Management System also allows for creation of cumulative as well as quality control reports.

The types of test cards available for use with the VITEK® 2 Compact system are as follows:

- Gram-Positives—The GP card (or GPI card) is designed for identification of Gram-Positive cocci, staphylococci, streptococci, enterococci, and related genera. Time to result is 2–8 h.
- Gram-Negatives—The GN card (or GNI+ card) is designed for identification of Gram-negative bacilli, and fermenting and nonfermenting bacteria. Time to result is 2–10 h. With the newer system, there is no longer the need for a separate non-fermenter Card (NFC).
- *Bacillus*—The BCL card is designed for identification of Gram-positive spore-forming bacilli. Time to result is 14 h.
- Yeasts—The YST (or YBC) card is designed for identification of yeasts and yeast-like microorganisms. Time to result is 18 h.
- Anaerobes—The ANC Card launched in February of 2008, is designed for on-line identification of anaerobes plus the most commonly isolated *Corynebacterium* spp. Time to result is 6 hours. The Card is incubated in the system rather than in an offline incubator as with the 1st generation system.
- Neisseria card—The NH card is designed for identification of the *Neisseria, Haemophilus,* and other fastidious species. It is also capable of identifying *Campylobacter (fetus and jejuni), Capnocytophaga* sp., *Cardiobacterium hominis, Eikenella corrodens, Gardnerella vaginalis, Kingella* sp., *Moraxella (Branhamella) catarrhalis, Oligella urethralis,* and *Suttonella indologenes.*

Other Cards are also available for purposes other than identification of microorganisms: The BIO Card (Bioburden), seen in Figure 9.7, can be used with the VITEK® system for the automated enumeration of microorganisms in a liquid sample, and numerous types of Susceptibility Cards (e.g., VRE, AMP C, MRSA, and Natural Resistance) are also available for diagnostic testing.

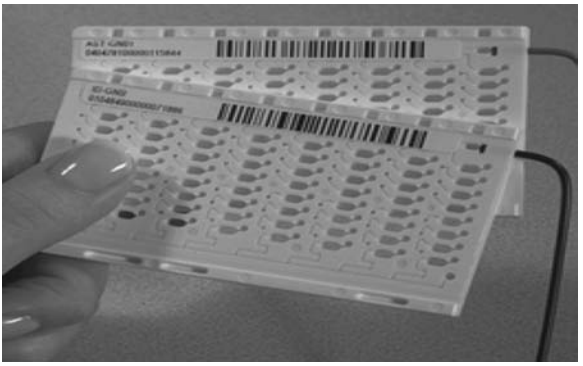


FIGURE 9.7 Bioburden Cards used with the VITEK® System. (Courtesy of bioMérieux, Inc. www.biomerieux-usa.com. With permission.)

The Bioburden Card

The Bioburden Card is intended for automated enumeration of microbial populations in a liquid sample. A wide range of aerobic as well as facultative anaerobic microorganisms can be detected with this test kit. This Card estimates microbial populations by the dilution method; results are given in MPN terms. Each of the 30 wells in the test Card contains a general-purpose growth medium that promotes the growth of fastidious and nonfastidious organisms. The liquid sample is inoculated into the card and placed in a built-in 35°C incubator equipment chamber. The system optically scans the Card each hour to detect the presence or absence of growth in each of the 30 wells based on light attenuation measured by an optical scanner. The total number of positive wells and the time required for positive reactions to occur determine the population estimate (MPN). Although user-friendly, applications of this test kit are limited to clear samples because any product turbidity or coloration may reduce the sensitivity of the test.

BioMérieux, Inc., also makes semiautomated bacterial identification systems such as the **ATB® System**, which has three major components: the hardware, the software, and the disposable test strips. The system's hardware has a densitometer designed to measure the bacterial density obtained in an ampoule of liquid medium, a semiautomatic reader designed for reading ID 32 and rapid ID 32 strips, a computer designed to interpret the data generated, semiautomatic pipettes designed for aspiration and homogenization of liquids to be inoculated into the test strips, and a printer. The software is designed to interpret biochemical profiles.

The disposable test strips have cupules containing dehydrated substrates for biochemical tests. Test strips are available for the identification of Enterobacteriaceae (rapid ID 32E, 4 h test); streptococci and other microorganisms (rapid ID 32; 4 h test); anaerobes (rapid ID 32A, 4 h test); yeasts (ID 32C); Gram-negative rods (ID 32GN), which cannot be used manually; and staphylococci and micrococci (ID 32 Staph).

After inoculation with a suspension of the unknown organism, the reactions produced during incubation result in colorimetric or assimilation changes, which can be spontaneous, or created by addition of reagents or increase of turbidity. Results can be read by the automatic reader or manually. The test results are then either automatically or manually transferred to the computer for generation of a biochemical or numerical profile, which can be compared to known profiles for an identification match.

The Biolog Systems

The Biolog microbial identification (ID) systems (Biolog, Inc. www.biolog.com) are based on redox chemistry to perform carbon utilization tests for bacteria and fungi during metabolism. These systems use standardized 96-well MicroPlates™ each containing 95 different carbon sources and one nutrient control well, in the form of pre-filled and dried substrates ready for inoculation with a suspension of the test organism. The test protocol is simple and fast. A pure culture of the organism is prepared at a specified cell density and inoculated into the MicroPlate™ which is then incubated at specified conditions (online or offline). For most bacteria, the test kit incubates between four hours to overnight. For yeasts and other slow-growing organisms, a longer incubation period may be needed. Utilization of a carbon source

FIGURE 9.8 The Omnilog Microbial Identification System. (Photo courtesy of Biolog, Inc., www.biolog.com.)

is detected (visual or via the system's reader with dual wavelength detection) as an increase in respiration of cells in the reaction wells, leading to irreversible reduction of a tetrazolium dye. A positive reaction is indicated by a purple color. MicroPlates™ used for identification fungi contain assimilation tests that give turbidimetric rather than colorimetric responses. Therefore, it is recommended that the automated reader be used in cases where visual readings may be difficult.

Microorganisms are identified based on their characteristic "metabolic fingerprints" (respiration), a unique pattern because it is based on over 200 genes responsible for the metabolic processes analyzed. The resulting metabolic pattern is recorded and compared to the profiles of hundreds to thousands of organisms in the systems' databases for the final microbial identification result. Biolog's databases contain over 2,000 species of bacteria, yeast and filamentous fungi and they are: GP database (gram-positive aerobic bacteria); GN database (gram-negative aerobic bacteria); AN database (anaerobic bacteria); YT Database (yeasts); and FF Database (filamentous fungi).

Biolog, Inc. offers manual, semi-automated and automated microbial ID systems. For the manual systems, the user must read the reactions in each of the test

wells visually and then input the results into the system's software data entry screen for comparison and organism identification. The **MicroLog™ 1** manual system only print reports while the **MicroLog™ 2** manual system has additional capabilities for data saving, data management and creation of customized databases. Both systems can be upgraded for semi or full automation as the needs of the user company evolve.

The **MicroStation™** is a semi-automated ID system consisting of a plate reader, software, and databases (all six Biolog databases). For this system, the user incubates the MicroPlates™ off-line and then places them in the MicroStation™ Reader for analysis. This system is capable of identifying aerobic gram-positive and gram-negative bacteria, anaerobic bacteria, yeasts and molds (filamentous fungi).

The **OmniLog®** ID System, shown in Figure 9.8, is fully automated with integrated incubator and plate reader, software and databases. This system can only process GN and GP MicroPlates™. However, the **OmniLog® Plus**, a MicroStation™ plus an OmniLog®, adds testing capability for anaerobic bacteria and fungi. The latest breakthrough in redox chemistry technology is used with the **OmniLog GEN III** which offers many testing advantages to include testing of both Gram-positive and Gram-negative bacteria in the same test panel (no need for Gram stains or pretests) and an expanded database.

The Biolog microbial ID systems generate reports containing strain-specific biotype patterns, species identification and other valuable information, such as dendograms. Reports can be exported to popular software formats such as Excel® and Access®. Refer to Figure 9.9 for a sample report generated by the Omnilog® System. Other features include online organism information so that the microbiologist can find out more about unfamiliar species. The software used with the Biolog systems also provides for data management and data storage capabilities and allows the user to create customized databases for epidemiological evaluation or for other purposes.

Fatty Acid Analysis Using Gas Chromatography

One of the artifact-based (phenotypic) technologies used for microbial identification makes use of gas chromatography to determine the fatty acid composition of microbial cell walls, which is very stable and conserved. A pure suspension of an unknown organism is saponified and methylated, and fatty acids are extracted using traditional chemical procedures. The prepared sample is then loaded onto a gas chromatography (GC) system and analyzed. The fatty acid profile chromatogram obtained is compared with the fatty acid profiles of known organisms contained in the system's database, and an identification result is generated. One of the systems that use this technology is the **Sherlock® Microbial Identification System** (MIDI, www.midi-inc.com), which is discussed next.

The MIDI System

The Sherlock® Microbial Identification System (MIS), shown in Figure 9.10, uses GC to analyze cellular fatty acid methyl esters. Using pattern recognition, the fatty acid profile of the unknown organism is compared to the fatty acid profiles stored in the database, producing a microbial identification. This system has been used worldwide

ProgramName	OmniLog 2.1.01.136
ProjectName	OL3
DataFileName	OL3_196_080418_N.D5E
DataPath	C:\Documents and Settings\Administrator.TAS-3749COH\WF1F\Desktop
WSOperator	J Smith
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Apr 17 2008 2:57 PM
Maximum Incubation Hours	22
Current Incubation Hours	22.00
Reader	196
Position	20B
PlateType	GEN III
Protocol	A
Sample ID	648993E
Sample Source	BUILDING 45
Notes	RETEST
MicroPlate Lot No.	2387649
Inoculation Fluid Lot No.	098747623
ID Date Time	Apr 18 2008 1:00 PM
Biolog ID DB	C:\Program Files\Biolog\OL_DB_dir\databases\Biolog GEN III DB.I5G
ID State	Final ID
ID Result	Species ID: Escherichia coli
ID Organism Type	GN-Ent
ID Comment	
ID Notice	

Rank	PROB	SIM	DIST	Gram	Organism Type	Species
1	1.000	0.841	2.280	GN	GN-Ent	Escherichia coli
2	0.000	0.000	7.216	GN	GN-Ent	Escherichia coli inactive
3	0.000	0.000	9.123	GN	GN-Ent	Escherichia coli O157:H7
4	0.000	0.000	9.894	GN	GN-Ent	Escherichia fergusonii

Report Date Apr 18 2008 1:10 PM

FIGURE 9.9 Sample report generated by the Omnilog System. (Photo courtesy of Biolog, Inc., www.biolog.com.)

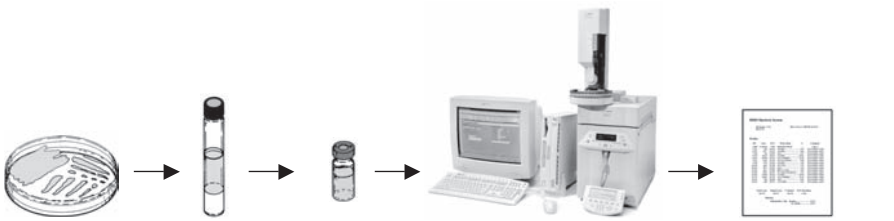


FIGURE 9.10 The Sherlock® Instant FAME procedure. (Photo courtesy of MIDI, www.midi-inc.com. With permission.)

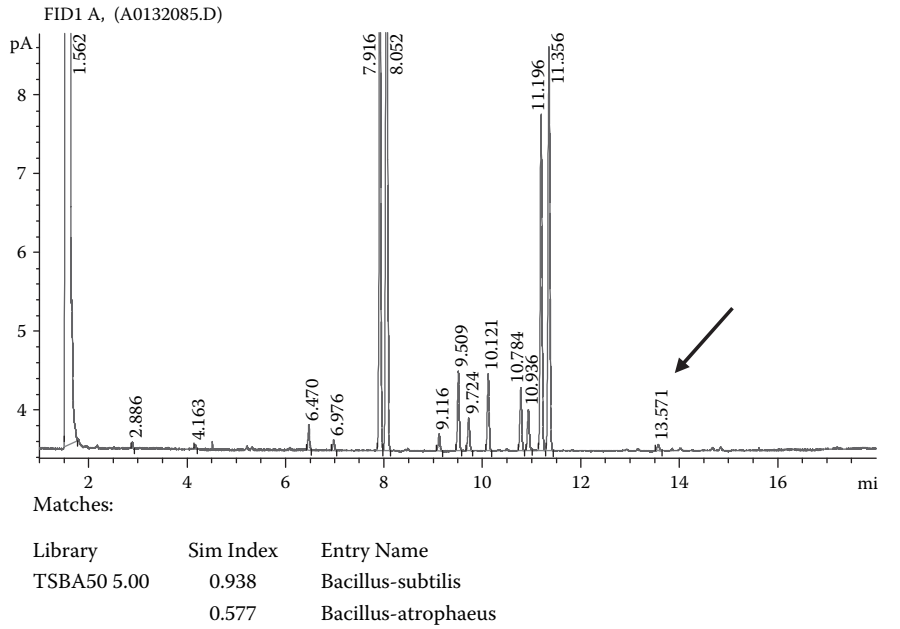


FIGURE 9.11 Sample report generated by the MIDI for *Bacillus subtilis* sample, using the standard chromatography method. (Photo courtesy of MIDI, www.midi-inc.com. With permission.)

since 1985 in clinical and environmental laboratories, and it is capable of identifying over 1500 aerobic and anaerobic bacterial species, and over 190 yeast organisms.

Although the principle of the technology and system used is based on analytical chemistry, the system was developed for microbiologists; hence, experience with chromatography techniques is not needed. However, the analysis does require a high degree of standardization using calibration standards, to include known pure cultures.

The **Sherlock® Instant FAME** (Fatty Acid Methyl Ester) new sample preparation has also minimized chemical manipulations of test samples, and transmethylation can now be easily accomplished in a simple 3-min procedure.

The MIDI Sherlock operates exclusively on Agilent® Technologies' GCs. The system is equipped with a flame ionization detector, an autosampler with injector, a controller and tray, a computer running Windows XP or Windows 2000, and the Agilent Chemstation Software. The GC is jointly controlled by Sherlock and ChemStation software to calibrate and monitor the system, ensure proper functioning, integrate the peaks, perform analyses, compare the peaks' retention times to the MIDI database, and print out sample identification information. Using advanced electronic control features of the Agilent® GCs, results are obtained in a fraction of time as compared to standard chromatographic methods (see Figures 9.11 and 9.12). The fatty acid profiles of organisms can also be presented as a dendrogram to show similarities with other organisms at the species, subspecies, and strain levels. This is a very useful tool in pharmaceutical microbiology for trend studies and for evaluation of possible sources of contamination. The MIDI Sherlock also allows the user

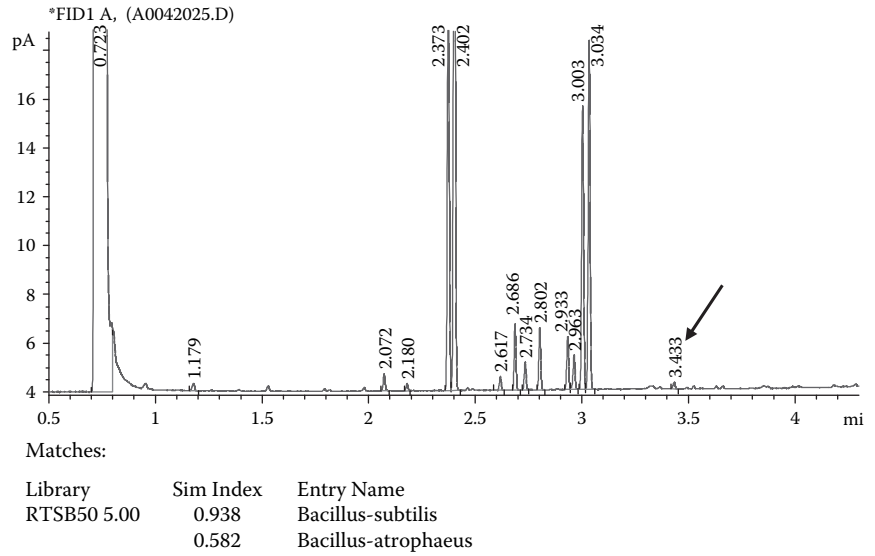


FIGURE 9.12 Sample report generated by the Sherlock® Rapid Methods for *Bacillus subtilis* sample. (Photo courtesy of MIDI, www.midi-inc.com. With permission.)

to create a customized database for identification results from environmental and product isolates.

Perhaps the main limitation of the FAME technology is the fact that fatty acid profiles of some related species are too similar to be definitively identified. In families where species are closely related, as in the case of Enterobacteriaceae for which taxonomy has been based mainly on biochemical reactions, the MIDI library entries of "closely related species" show some overlap with close second and third choices. However, when using a combined reporting of Sherlock FAME–DNA, this limitation is eliminated, and an accurate result can be obtained. This topic is addressed later in this chapter.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs have been used quite extensively over the years in diagnostic testing for detection and quantitation of antibodies and antigens. In a simple ELISA, the antigen is affixed to a surface (usually a microtiter plate) and coated with a specific antibody that is linked to an enzyme. This procedure results in an antigen–antibody complex, which is then presented with a substance that reacts with the enzyme to generate some type of detectable signal. For example, when performing a fluorescence ELISA, the antigen–antibody complex will fluoresce in the presence of light, and the amount of light generated can be correlated to the amount of antigen in the sample. There are various systems on the market for detection of foodborne pathogens and their toxins, as well as for clinical microbiology applications, to include the **Assurance EIA** (BioControl, www.biocontrolsys.com), **Tecra Opus** products (Biotrace International, www.biotrace.co.uk/), the **Salmonella-Tek ELISA** (Orga-

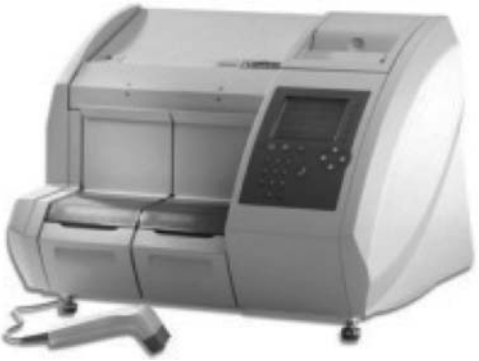


FIGURE 9.13 The Mini VIDAS® automated system for the screening of pathogens. (Photo courtesy of bioMérieux, Inc., www.biomerieux-usa.com. With permission.)

non-Teknika, a division on bioMérieux, Inc., www.biomerieux-usa.com), and the VIDAS® (bioMérieux, Inc., www.biomerieux-usa.com).

The VIDAS

The **VIDAS** is a fully automated enzyme-linked fluorescent immunoassay (ELFIA) technology, a version of the well-known ELISA technology, which uses 4-methyl umbelliferyl phosphate as the fluorescent substrate. The VIDAS is used for screening of pathogens such as *Salmonella*, *Listeria*, *Escherichia coli* O157, *Listeria monocytogenes*, and *Campylobacter* and for detection of toxic products such as Staphylococcal enterotoxins A–E. The end result of the testing protocol is a fluorescent product. The VIDAS Reader, using a special optical scanner, measures the amount of fluorescence generated in the assay.

The system's components include the following:

- VIDAS Reader module
- Controller microprocessor
- Computer
- Printer
- Data terminal
- Disposable assay kits

Each assay kit contains all the materials needed to run a specific assay, reagent strips, and solid-phase receptacles (SPR). The sealed reagent strip contains ten wells with predispensed reagents. The first well is empty, which is where the sample is placed. The next eight wells contain immunoassay reagents or washes. The last well is an optical cuvette, where the substrate reaction is measured for its fluorescent reading. The system allows for multiple samples to be processed at one time. The **mini VIDAS**, shown in Figure 9.13, is a compact and fully automated system. Both the VIDAS and the mini VIDAS are ISO certified and have been successfully used in the pharmaceutical industry as a substitute for the conventional screening tests for pathogens such as *Salmonella*.

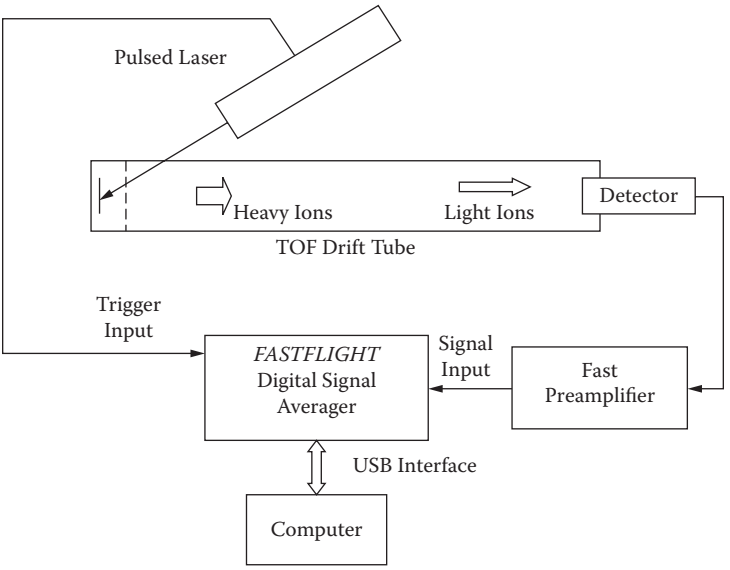


FIGURE 9.14 Schematic of the MALDI-TOF-MS technology.

Analysis of Biomolecules Using Mass Spectrometry

Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample, and it has been used for microbial identification using characteristic spectra of gaseous breakdown products generated when microbes are exposed to a heat source. Lately, microbial identification has been performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), a technology based on soft ionization of sample molecules using a laser beam (typically a nitrogen laser), which excites the molecule, causing it to give up an electron.

During MALDI-TOF-MS analysis, the sample is mixed with a matrix consisting of crystallized molecules (e.g., sinapinic acid or 2,5-dihydroxybenzoic acid) to facilitate vaporization and ionization, and most importantly, to protect the molecules from being destroyed by the laser light. The sample preparation is then spotted onto a MALDI plate. As the solvents used to prepare the sample vaporize, they leave behind a cocrystallized MALDI-TOF spot (analyte and matrix). When the laser is fired at the MALDI-TOF spot, the energy is absorbed by the matrix, which, then, transfers part of it to the analyte. In this manner, the matrix protects fragile molecules from the direct ionization source. Sample analysis is performed using a time-of-flight (TOF) analyzer, which measures the time it takes for the ions to travel through a region in the detector that is free of electrical fields. This region in the system is called "the flight tube" (see Figure 9.14). The TOF analyzer separates ions according to their **mass-to-charge (m:z)** ratios. Heavier ions are slower to cross the flight tube as compared to lighter ones. Because MALDI-TOF uses an ionization technique that protects the molecules from direct laser bombardment, it has been successfully used to analyze biomolecules such as peptides, proteins, glycoproteins, oligosaccharides, and oligonucleotides.

During the past few years, MALDI-TOF-MS has been used to analyze microbial suspensions for biomarkers (e.g., proteins, lipids, carbohydrates, etc.) and the generation of unique spectral fingerprints that could be used for identification of microorganisms at the species and even strain levels. As with other automated microbial identification systems, the spectra generated are compared to those contained in a mass spectral biomarker database. The **Spectral Archive and Microbial Identification System** (**SARAMIS™**) (AnagnosTec, www.anagnostec.de/) is a system that has been designed for characterization of microorganisms based on the MALDI-TOF technology. The mass spectra generated are compared with the spectra of well-known microorganisms that contain specific biomarkers characteristic of certain groups of microorganisms. According to the vendor, more than 1600 spectra for microorganism identification are available in the system's database.

Most of the published articles indicate that although promising, MALDI-TOF-MS technology needs some refinements to increase the accuracy of test results. As indicated by a study performed to analyze the spectra of fragmented rRNAs in comparison with database of characteristic microbial oligoribonucleotides, the MALDI-TOF-MS seems to be a very promising technique for microbial identification. However, better methods are needed in order to improve distinctions between the nucleotides uracil and cytosine, and the performance in general, perhaps by using the subregions of RNA in the analysis [10].

Although promising for microbial identification procedures, MALDI-TOF-MS has not yet become a routine procedure in QC microbiology laboratories. This is primarily because there are few standardized protocols for the development of a database with reproducible protein profiles from a broad range of microorganisms, and also due to the high cost of mass spectrometry systems as compared to other microbial identification systems that have proved reliable over the years.

Polymerase Chain Reaction (PCR)

Microbial identification using automated gene sequencing has proved to be a powerful tool to microbiologists. In fact, the microbial taxonomy found in the latest edition of Bergey's *Manual of Systematic Bacteriology* is based on the 16S ribosomal (r)RNA gene sequence data. The use of this technology for microbial identification is also mentioned as a preferred method in the FDA Guidance for Industry—Sterile Drug Products Produced by Aseptic Processing [11], as well as in the USP Chapter <1117>, *Microbiological Best Laboratory Practices.* This is because DNA sequencing has proved to be a more accurate and reproducible method for microbial identification as compared to phenotypic and biochemical methods.

Microbial identification using PCR technology is based on sequence data for the rRNA gene as it is the most conserved gene in cells, meaning that the rRNA gene has essentially remained unchanged throughout evolution.

A ribosome, made up almost entirely of rRNA, is a cytoplasmic particle that is part of the protein-manufacturing machinery of all living organisms. Its function is to provide a mechanism for translating messages contained in the mRNA into amino acids, the building block of proteins. Each ribosome has two subunits, and these are very similar in prokaryotes and eukaryotes.

Prokaryotes have 70S ribosomes, each composed of a small (30S) subunit and a large (50S) subunit. The letter "S" denotes their different sedimentation characteristics expressed in Svedberg (S) units. The large subunit consists of one 5S RNA strand, one 23S RNA strand, and 34 proteins. The 30S subunit is made up of a single strand of RNA (16S rRNA) bound to 21 proteins. The conserved and ubiquitous 16S rRNA gene is relatively short (1.5 kb), thus making it an excellence molecular marker to sequence.

Eukaryotes have 80S ribosomes, each composed of a small (40S) subunit and a large (60S) subunit. The large subunit consists of one 5S RNA strand, one 28S RNA strand, one 5.8S subunit, and about 49 proteins. The smaller 40S subunit has one 18S RNA and about 33 proteins. Microbiologists prefer to use the 28S rRNA gene as a molecular marker for identification of fungi because sequencing of the 18S rRNA gene often does not provide sufficient taxonomic resolution to allow for accurate fungal identification to genus or species levels in mixed communities. Eukaryotes also have ribosomes in chloroplasts and mitochondria that are very similar to prokaryotic ribosomes; according to the endosymbiotic theory, these ribosomes are descents of bacteria that once lived inside eukaryotes in a symbiotic relationship, and were then absorbed by the eukaryotic cells and became part of their cellular machinery.

The procedure for gene sequencing starts with isolation of DNA from a pure culture, amplification of the 16S rRNA gene (bacteria and archaea) or 28S rRNA gene (fungi), and followed by sequencing the gene using a genetic analyzer. The sequence data obtained is then compared to a database library containing hundreds to thousands of known 16S rDNA and 28S rDNA sequences. The system then provides a percent match or percent identity to the closest matches based on pairwise alignment and phylogenic trees. Pairwise alignment is comparison of two sequences while allowing certain mismatches between them. This type of analysis is performed to achieve the same length and to display maximum similarity/conservation on a character-by-character basis.

Representative systems on the market for microbial testing using the DNA sequencing technique include the **MicroSeq® Microbial Identification System** (Applied Biosystems, www.appliedbiosystems.com) and the **Sherlock DNA** (MIDI, www.midi-inc.com). Bacterial libraries are based on sequencing data for the 16S rRNA gene either in full length (1542 base pairs) or in just the first 500 base pairs. The fungal library is based on sequencing data for the 28S rRNA gene.

The **Sherlock DNA** has a unique feature as samples can be run using both the FAME and PCR technologies to achieve the most accurate results. In the example shown in Figure 9.15, both the FAME and DNA results are ambiguous. However, the combined *polyphasic disambiguation report* is able to produce an identification result with good confidence level.

Detection of Microbial Contamination Using PCR Technology

The **BAX® Detection System** (Dupont Qualicon, www2.dupont.com/Qualicon/en_US/) is a fast and accurate method for detecting microorganisms in product and environmental samples, using PCR to amplify, to detectable levels, a very specific genetic sequence that is unique to a given organism. The system uses real-time or

Sample ID: DBLDIS-SEQV6-11 (17-Rapid)

FAME Matches:

Library	Sim Index	Entry Name
RTSBA6 6.10	0.901	Methylobacterium-mesophilicum/radiotolerans
	0.896	Xanthobacter-flavus
	0.822	Methylobacterium-rhodesianum-GC subgroup A/zatmanii
	0.672	Methylobacterium-rhodesianum-GC subgroup B
	0.664	Actinomadura-oligospora
	0.627	Rhodobacter-sphaeroides
	0.575	Methylobacterium-organophilum/fujisawaense

DNA Matches:

Match	%Diff	Length	Library Entry Name
1	0.00	471	Methylobacterium-radiotolerans
2	0.85	471	Methylobacterium-fujisawaense
3	2.76	471	Methylobacterium-mesophilicum
4	3.59	473	Methylobacterium-rhodinum
5	3.80	473	Methylobacterium-extorquens
6	3.80	473	Methylobacterium-zatmanii
7	4.22	473	Methylobacterium-rhodesianum
8	4.25	471	Methylobacterium-organophilum

Cross Library Report:

%Diff	Genus	Species	FAME SI
0.00	**methylobacterium**	**radiotolerans**	**0.901**
0.85	methylobacterium	fujisawaense	0.575
2.76	methylobacterium	mesophilicum	0.901
3.59	methylobacterium	rhodinum	0.020
3.80	methylobacterium	zatmanii	0.822
3.80	methylobacterium	extorquens	0.267
4.22	methylobacterium	rhodesianum	0.822
4.25	methylobacterium	organophilum	0.575
11.21	xanthobacter	flavus	0.896
11.89	rhodobacter	sphaeroides	0.627
19.64	actinomadura	oligospora	0.664

FIGURE 9.15 Polyphasic microbial identification report generated by FAME and PCR technologies. (Photo courtesy of MIDI, www.midi-inc.com. With permission.)

end-point analysis to determine the presence or absence of microorganisms, and delivers LIMS-compatible results within 90 min to 4 h, depending on the test. The BAX Detection System has been used by many companies around the world as an integral part of their quality control systems owing to its capability of decreasing false positives, minimizing retesting, reducing employee training, and speeding the time to market.

Pulse-Field Gel Electrophoresis (PFGE)

PFGE is a technique used to separate DNA, especially long strands, for the purpose of performing genetic subtyping. This technique involves alternating electric fields to run DNA through an agarose gel and the use of highly specialized equipment. DNA from organisms purported to be of the same subtype/source are analyzed

FIGURE 9.16 The Riboprinter® Microbial Characterization System. (Photo courtesy of DuPont Qualicon, www2.dupont.com/Qualicon/en_US/. With permission.)

by the equipment software. PFGE has proved to be a useful tool for differentiating between isolates belonging to the same or closely related species, for tracking the distribution of specific strains in the environment, and for determining sources of microbial contamination. A case study performed at Molecular Epidemiology, Inc. (www.molecularepi.com), to investigate a microbial contamination event is presented at the end of this chapter. In this study, scientists used a polyphasic approach to microbial identification, which included PFGE technology.

Riboprinting

Riboprinting (Ribotyping) is a method of genotyping microbial isolates based on the Southern Blot analysis, which utilizes a labeled ssDNA probe from the 16sRNA codon. The process is fully automated, and it starts with the lysing of the cells to release the DNA, and then cutting the released DNA into fragments using restriction enzymes. These DNA fragments are then separated by size using gel electrophoresis and transferred to a membrane, where they are hybridized with a DNA probe and coated with a chemiluminescent agent. A digital camera captures the image from the gel and converts the luminescing DNA bands to digital information. The DNA pattern generated is then compared with other patterns of known organisms in the database for characterization. This method is useful to perform strain differentiation when the microbial species is known or is not relevant.

The Riboprinter®

The **RiboPrinter® Microbial Characterization System** (Figure 9.16) of DuPont Qualicon (www2.dupont.com/Qualicon/en_US) is a fully automated riboprinting

system. It produces genetic fingerprints based on a microorganism's rRNA genes in about 8 h.

The first step in the fingerprinting process involves extraction and restriction digestion of DNA from the microorganism in question. This is accomplished by using a simple colony pick to collect growth from a pure culture on an agar plate, which is then suspended in a buffer and heat treated in the sample carrier. The carrier is then transferred to the instrument, and all subsequent steps are fully automated. In the characterization unit, cells are lysed to release DNA, which is then digested to completion with a restriction enzyme. The resulting DNA restriction fragments are transferred to an agarose gel cassette containing 13 wells. Using a marker DNA and direct blot electrophoresis, the DNA fragments are separated by size and transferred to a moving nylon membrane. After denaturation, each membrane is hybridized with a chemically labeled rRNA operon from *Escherichia coli*. Additional treatments make each electrophoresis band containing the rRNA genes visible to a customized CCD camera located in the system. The detected light intensity is converted to digital information and transferred for automated software analysis. The RiboPrinter system statistically compares the sample pattern with the patterns from previously processed samples or with the existing patterns in the instrument's library. The system then produces a report, presenting the characterization and identification for each sample. This information can be used in a variety of applications ranging from the tracking of contamination sources to research and development. Figure 9.17 summarizes the ribotyping process. Figure 9.18 is a sample report generated by the RiboPrinter®.

Fluorescent Labeling Assays

Fluorescent labeling has been used for real-time (usually 2 h) enumeration of microorganisms in food, water, and pharmaceutical products. The automated systems available on the market use fluorescent vital dyes to label viable microorganisms for detection and enumeration. After the microbial cells have been labeled, the samples are exposed to a laser beam. The system then scans and counts the fluorescing cells. This type of technology eliminates the need for the microbial growth step, and offers improved test sensitivity with capability of detection of a single cell.

Some systems use a solid-phase cytometry (SPC) laser scanning technique, where filterable samples are collected on a membrane filter, and any viable microbial cell captured on the filter is stained with a vital stain. The membrane filter is then scanned with a laser beam to enumerate the fluorescing viable cells. Other systems use flow cytometry, a technique that simultaneously measures and analyzes cells in a fluid stream as they pass by a beam of light. Flow cytometry is applicable for filterable as well as nonfilterable samples.

Scan® RDI Microbial Detection

The Scan® RDI (AES-Chemunex, www.aeschemunex.com), shown in Figure 9.19, uses solid-phase cytometry for detection and enumeration of microorganisms. This system is capable of direct labeling of individual microbial cells without the need for a growth phase. The test kit contains either viability markers (Fluorassure™

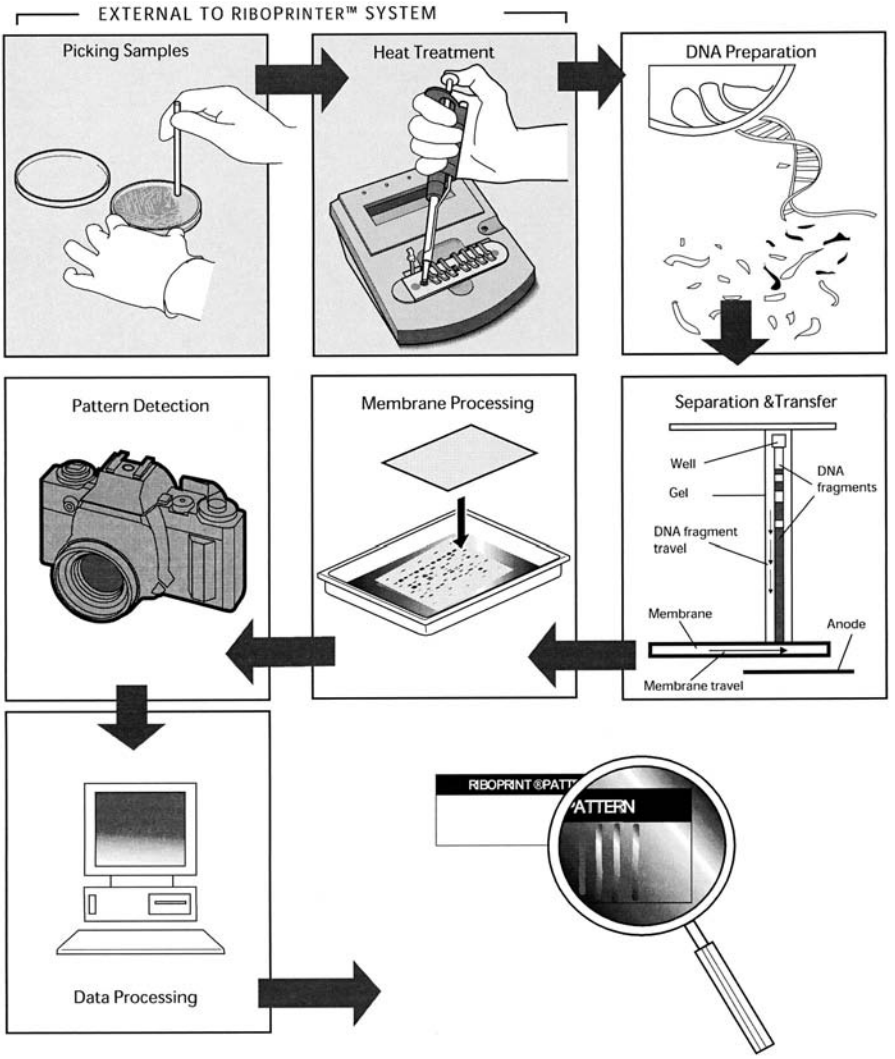
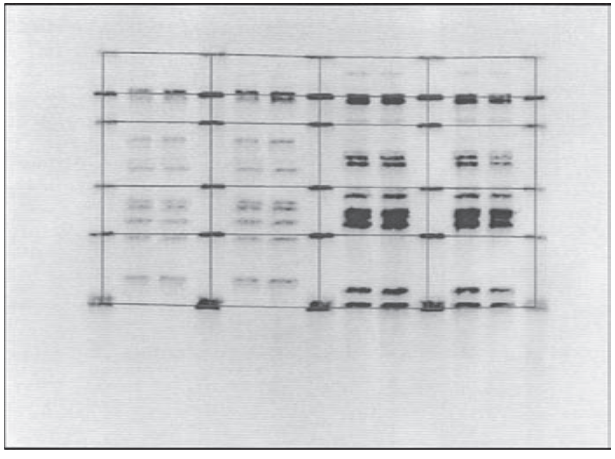


FIGURE 9.17 The ribotyping process. (Photo courtesy of DuPont Qualicon, www2.dupont.com/Qualicon/en_US/. With permission.)

Process Start Step	Normal
Batch Comment	New install-QC
Batch	275-377
Operator	TRB
S/T Unit Number	1
Started	6/27/2007 11:52:33 AM
Conjugate Lot	6290
DNA Prep Enzyme Lot	×
DNA Prep Lot	6290
Gel Cassette Lot	7004
Membrane Lot	6342
MP Base Lot	6300
Probe Lot	6290
Substrate Lot	6290
Adjusted Exposure Time	25.29
In-Lane Normalized	30.70
Non-Lane Background Normalized Signal	2.28
Signal To Background	13.45



	Sample Number	Normalized Background	Normalized Signal	Signal to Background	DuPont ID	DuPont ID Label	DuPont ID Similarity	RiboPrint™ Pattern 1 kbp 5 10 15 50
1	275-377-S-1	2.84	2.78	0.98	QC-204	Bacillus pumilus	0.98	
2	275-377-S-2	3.18	3.46	1.09	QC-204	Bacillus pumilus	0.97	
3	275-377-S-3	3.92	3.86	0.98	QC-204	Bacillus pumilus	0.97	
4	275-377-S-4	3.98	5.47	1.38	QC-204	Bacillus pumilus	0.97	
5	275-377-S-5	4.25	9.83	2.31	DUP-14591	Enterobacter aerogenes	0.97	
6	275-377-S-6	4.43	9.78	2.21	DUP-14591	Enterobacter aerogenes	0.97	
7	275-377-S-7	3.52	9.38	2.66	DUP-14591	Enterobacter aerogenes	0.96	
8	275-377-S-8	3.07	7.43	2.42	DUP-14591	Enterobacter aerogenes	0.96	

FIGURE 9.18 Sample report generated by the Riboprinter® system. (Photo courtesy of DuPont Qualicon, www2.dupont.com/Qualicon/en_US/. With permission.)

FIGURE 9.19 The Scan® RDI microbial detection system based on solid phase cytometry. (Photo courtesy of AES-Chemunex, www.aeschemunex.com. With permission.)

reagents) for total aerobic microbial count and total fungi count, or specific microbial identification markers (probe-based markers) for detection of specified microbial species (pathogens). The fluorescent labeling technology used by Chemunex is capable of distinguishing between live and dead cells, as only metabolically active cells are able to enzymatically cleave the initially nonfluorescent dye to liberate free fluorochrome and retain the fluorescent label within the cell. The Scan® RDI uses a four-step protocol:

1. First, the sample is filtered through a 25-mm membrane filter.
2. Then, the filter receives the fluorescent dye to label any microbial cell retained on the filter.
3. In the third step, the membrane filter is scanned with a laser beam, and viable cells are detected and enumerated.
4. The last step, which is optional, involves visual confirmation of detected microbes using a fluorescent microscope.

A sample image generated by the Scan RDI is shown in Figure 9.20.

At the New Technologies Forum 6: Rapid Methods in Microbiology, held at the Royal Pharmaceutical Society in February, 2003, many of the rapid microbial technologies were discussed in detail. Bob Johnson from Pliva, a member of the Barr Group located in Zagreb, Croatia, mentioned that "The Scan® RDI" is the only current technology with the sensitivity and near real-time results to support in-process testing … and support the ideas of PAT and parametric release." He also stated that when this instrument was used to detect and enumerate biofilm cells from in vivo and in vitro experiments, higher counts were obtained as compared to a standard plate count method. Bob Johnson concluded that the Scan® RDI "was a more sensitive technology."

D-Count

Chemunex also developed the D-Count microbial detection system based on flow cytometry, which is able to process nonaqueous materials (see Figure 9.21). This system incorporates the same fluorescent labeling technology used for the Scan® RDI, but because the sample is in suspension, the cells are scanned with a laser beam as they pass through a flow cell.

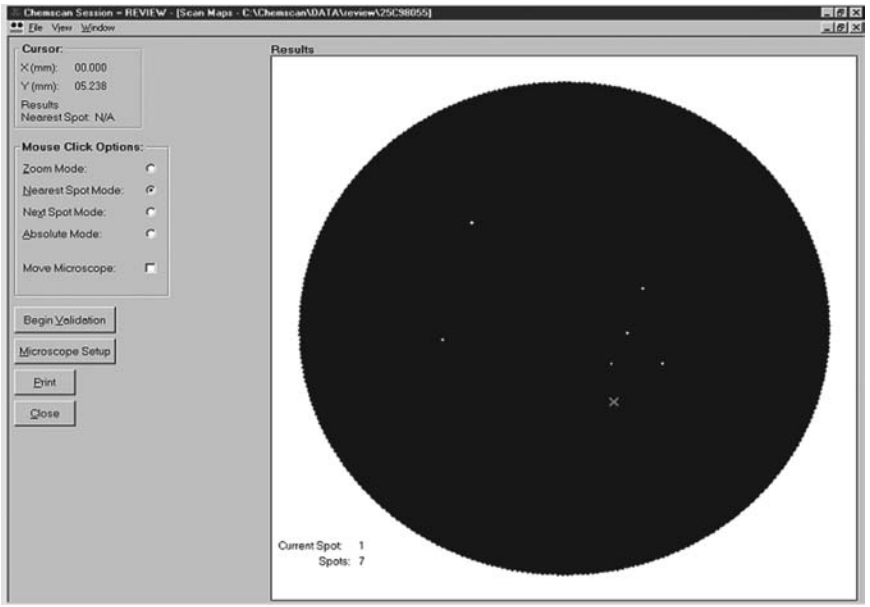


FIGURE 9.20 Image generated by the Scan® RDI microbial detection system. (Photo courtesy of AES-Chemunex, www.aeschemunex.com. With permission.)

FIGURE 9.21 The D-Count microbial detection system based on flow cytometry. (Photo courtesy of AES-Chemunex, www.aeschemunex.com. With permission.)

Biosensors and Microarrays

A new and exciting field in applied microbiology is the use of biosensors and biochips (microarrays) for detection and identification of microbial contamination. A biosensor is an analytical device based on the use of a biological material for its sensing function. When the biological component of the system reacts or interacts specifically with the analyte, the result is a chemical or physical change (signal) that is detectable. The signal can be electrochemical (e.g., impedance), optical (e.g., bioluminescence), or another type.

A biochip is essentially a collection of minituarized biosensors (microarray) that can perform hundreds or thousands of simultaneous tests, thus enabling researchers to screen large numbers of samples at one time (see Figure 9.22). This technology

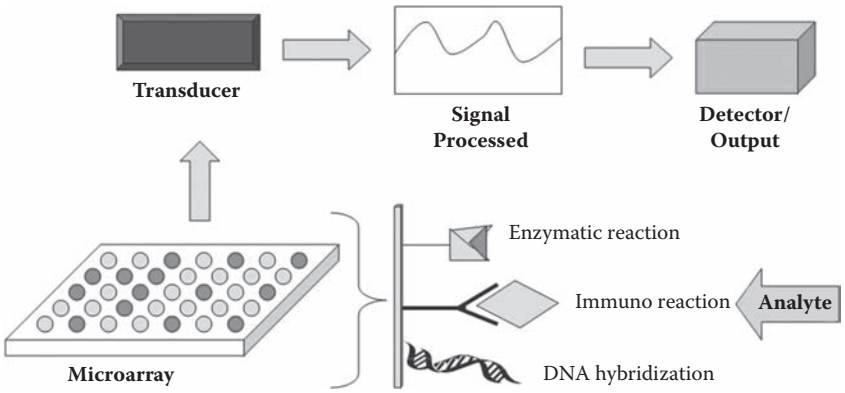


FIGURE 9.22 Diagram of biochip technology.

was developed as a tool to help improve the diagnosis, monitoring, and treatment of diseases. Other current uses of biochips or microarrays include analysis of the activity of specific genes during drug development and drug screening processes, and for detection of bioterrorism agents and human pathogens, the latter having possible applications in the field of microbial contamination of pharmaceutical products, foods, and environment. For testing of pharmaceutical products, a biochip can be specifically designed to simultaneously detect specified microbial species by imprinting on the chip a variety of antibodies or DNA molecules specific to the target pathogens.

One of the systems on the market that uses this technology is the **GeneChip®** (Affymetrix, www.affymetrix.com). This product consists of disposable DNA probe arrays containing selected gene sequences. The GeneChip is designed to detect low levels of specific pathogens in complex sample matrices and in samples with mixed microbial flora for species-level identification.

Although the technology is available, and there are products on the market that target pathogen screening, the use of biosensors and biochips for routine testing of pharmaceutical products is still at its infancy, and certainly more product development is needed to address users' concerns, such as test sensitivity and distinction between live and dead cells.

Laboratory-on-a-Chip Technology

Laboratory-on-a-chip (LOC) devices have emerged as an important technology platform for many areas of research, to include proteomics and clinical microbiology, because it provides for high throughput and on-site testing. Although this technology offers great potential for environmental testing and microbial identification, the use of LOCs has not been seriously considered for pharmaceutical microbiology applications. This is unfortunate because the possibility to perform in one miniaturized device sample collection, sample preparation, and simultaneous testing of various samples would be of great benefit to the pharmaceutical microbiologist. LOCs would be especially beneficial if used for antimicrobial effectiveness testing, disinfectant/sanitizer efficacy evaluations, for testing in support of equipment and facility clean-

ing validation, and during the collection of the multitude of samples when troubleshooting a microbial contamination event. However, given the fact that LOC is a new technology that was really not developed with pharmaceutical testing in mind, there are still some challenges that need to be overcome before it can be used to the benefit of pharmaceutical microbiologists. For example, targeted microbial species are often too dilute in the environment or sample materials to be readily detected in the required test volume without some amplification procedure. Until some of these technical issues are resolved, LOCs will most likely remain a tool for microbiologists working in method development and research laboratories.

BARRIERS TO IMPLEMENTATION

Rapid methods and alternate microbiology technologies have been on the market for many years, and despite the enormous interest in RMM by pharmaceutical microbiologists, companies are still reluctant to invest on these technologies. In the United States there is a lot of interest on alternate methods as evidenced by the numerous conferences, publications, and user groups. Yet, the level of adoption of RMM in this country is still much lower when compared to Europe. In fact, as pointed out during the 2003 New Technologies Forum 6: Rapid Methods in Microbiology, approval had already been granted by the MCA and EMEA for the use of the Chemunex equipment in Europe, whereas during the same period in the United States, the FDA had not received any applications for the use of RMM for microbial limits or sterility testing in support of product release.

So, what could be causing this reluctance to adopt RMM in the United States? Perhaps one could group the barriers to implementation of RMM into three categories: technical issues, company culture, and regulatory climate. Let us further analyze them separately:

Technical issues: Most alternate technology platforms were not designed with pharmaceutical testing in mind. Therefore, systems may not work for the types of pharmaceutical and biopharmaceutical samples that require microbial evaluation. In some cases, there is some lack of understanding of vendors on regulatory requirements and the testing needs. For example, some systems rely on destructive testing of microbial cells for detection and, therefore, if microbial contamination is detected, cells would not be viable for identification. There are also considerations over product interference, limit of detection, and method or equipment validation. The user must not only confirm that there is sufficient supporting data from the vendor to satisfy the regulators (e.g., software-compliant with 21 CFR Part 11) but also ensure that the equipment operates in a manner that will meet the scrutiny requirements for equipment validation.

Regulatory climate: Although there are regulatory guidance documents that address alternate technologies, there is still an uncertainty as to the acceptability of these methods for a particular application. One concern often raised by FDA investigators is the possibility that an alternate method could be more sensitive than traditional methods, thus leading to enhanced

microbial detection, which in turn could result in product/system to meet preestablished limits. In addition, there are still some gaps in terms of regulatory documents regarding certain technologies as well as in the training of investigators on RMM—lack of understanding of alternate technologies by regulatory reviewers could delay the approval of a new drug.

Company culture: Most companies have a conservative attitude when it comes to purchase of new equipment. First, there are business concerns in terms of cost and resources, that is, initial investment (to include high cost of equipment and validation work) and return on investment. Company managers are concerned that if they choose to invest on an RMM system, the validation work might not meet regulatory expectations. Therefore, companies are reluctant to commit resources and funds when there is the possibility that the alternate method may not be accepted by the regulatory agencies. Then, there is the conservative nature of the pharmaceutical industry in general when it comes to new technologies. This attitude certainly leads to a lack of managerial commitment to move away from traditional compendial testing and invest in rapid/alternate methods.

REGULATORY CLIMATE

As discussed in the preceding text, one of the barriers to introducing RMM in the pharmaceutical industry is the uncertainty on the part of pharmaceutical companies as to the acceptability of such methods by the various regulatory agencies. According to Dr. Bryan S. Riley from the FDA, CDER (PDA meeting, Milan, June 2003), the main concern expressed by the FDA over RMM is the increased sensitivity of RMM, which could change, perhaps raise, the microbial limits acceptance criteria, or lead to false positives in sterility testing. Even though there are still concerns over alternative technologies as applied to product release testing, the FDA has taken initiatives to address pharmaceutical manufacturing in the 21st century, and these should help address some of the users' concerns and facilitate the use of RMM. In fact, according to the presentations during the 2003 2nd Rapid Methods Users Group (RMUG; www.rapidmicro.org) conference held in Baltimore, Maryland, it became clear that the FDA is ready and willing to support rapid methods, and during the recent years, the agency has made several attempts to facilitate the approval of rapid methods. The PAT initiate has recommendations for users of RMM to have an open dialog with the FDA about the implementation of alternate analytical methods, to include microbiological methods. Another initiative that should help introduce RMM in QC microbiology laboratories is the creation of a dedicated and highly trained pharmaceutical inspectorate. This group of specialized investigators will include microbiologists trained and experienced in RMM. Last, but not least, the FDA's dispute resolution process, which allows for formal resolutions of disagreements that may arise during inspections, should facilitate discussions between RMM users and trained regulatory inspectors, thus helping clarify differences of opinion on these new technologies. Indeed, the best approach for submission of RMM to the FDA is to have a direct dialog with the agency and to ensure that a method comparability protocol, as discussed in Chapter 7 of this book, is included in the submission packet.

In Europe, as pointed out earlier, acceptance of RMM is greater, and the regulatory agencies have readily embraced alternative technologies. However, as pointed out in 2006 by the Rapid Microbiological Methods (RMM) Working Group, formed by the European Compliance Academy, European companies need regulatory clarity on the use of rapid methods because there is still a lack of standardization for submitting RMMs for approval.

Based on recent publications and discussions at national and international conferences, the author believes that there is a positive trend by the regulatory agencies to embrace the use of RMM not only for testing in support of pharmaceutical production but also for product release testing. There are some areas that users and vendors should focus on in order to improve understanding of RMM and expedite the regulatory approval process, and these include

- Companies should create scientifically sound validation packages reflecting the use of the technology at the user's site without relying solely on literature provided by vendors.
- In cases where there are differences in results as compared to traditional methods, companies should include in the validation reports reasons for these differences, and test acceptance criteria should be set according to these values.
- Vendors should invest in further product development with the focus on the needs of the pharmaceutical industry.
- Scientists at pharmaceutical companies and from vendor companies should carry out more research and publish more frequently on successful applications of RMM.
- RMM should be included as compendial methods instead of being listed as possible alternatives to traditional testing.

FUTURE TRENDS

There is no doubt that rapid methods in microbial testing are needed in today's pharmaceutical microbiology laboratories. Their use would provide for better control of manufacturing processes and operations, and increase efficiency and accuracy of testing. Based on the recent initiatives and guidance documents from the regulatory agencies and the compendia, it is safe to say that there is a positive trend in terms of acceptance of new microbiology technologies in the United States and abroad. This, in turn, may translate into a positive trend in implementation of such technologies by many pharmaceutical and biopharmaceutical companies. It is the author's prediction that priority will be given to automated microbial identification and detection systems that are based on genotypic methods. PCR technology has been in place for some time and has proven to be an indispensable tool for the pharmaceutical microbiologist. In addition, as discussed earlier, this technology has become the gold standard for microbial identification in support of product testing in the eyes of the FDA and the compendia. In terms of microbial detection and quantitation, the testing for total viable counts, total yeast and mold counts, and testing for specified microbial species will remain the primary tests for measuring microbial contamination in pharmaceutical products, processes, and raw materials. However, in order to

ensure product quality and safety, attention must be given to detection of fastidious and viable but unculturable organisms, to include biofilm cells. In addition, testing laboratories must find ways to improve efficiency and test turnaround times. These are key factors that will likely drive the implementation of RMM sooner than later. QC laboratory managers who choose not to embrace rapid methods will lag behind and miss on the opportunity to participate in quality improvement and cost reduction initiatives that would certainly benefit their companies. Hopefully, managers will reflect on the proven benefits of rapid methods and follow the trend seen in the food industry and in clinical microbiology. As there are so many different types of technology platforms, the microbiology manager must choose the best system that will ensure compliance with current regulations and expectations, and prove cost-effective and suitable for their application. Even if not used to replace approved methods for in-process or product release testing, alternative technologies can still be extremely helpful in expediting environmental monitoring test results as well as testing of clean utilities, disinfectants, and equipment change over samples.

In summary, the author believes that the future for rapid microbial methods is bright and we should all expect improvements in the various technologies discussed in this chapter to better meet the needs of pharmaceutical microbiology testing.

CASE STUDY: GENOTYPICALLY SIMILAR STAPHYLOCOCCI

(Contributed by Molecular Epidemiology, Inc., www.molecularepi.com)

Genetic-based identification techniques have been proposed as a powerful means to identifying microbial contaminants found in pharmaceutical manufacturing environments and products. Although such techniques are considered both robust and rapid, they are limited by several critical factors, including the quality and size of commercial databases. In some cases, genetic identification techniques using DNA sequencing of the 16S rRNA gene alone may not be able to adequately and appropriately place an unknown organism into its unique taxonomic position, thereby making it difficult to ascribe appropriate significance to its recovery from a particular site or product.

This case study, drawn from the pharmaceutical industry, demonstrates the use of a polyphasic approach, utilizing genetic identification techniques, classical phenotypic and morphologic methods, and combined with DNA fingerprinting (genetic subtyping) to provide a most reliable match to taxonomic classification. Such a thorough approach to microbial identification can be a powerful tool when performing root cause analysis during investigations of microbial contamination events.

Contaminant Isolate and Environmental Sampling

Five isolates were received by the reference laboratory from a pharmaceutical manufacturing company. Isolate A was recovered from a contaminated pharmaceutical product, whereas isolates B through E were recovered from routine environmental monitoring samples collected in the facility. These environmental isolates were gleaned from numerous other isolates that were recovered during the course of routine environmental monitoring of manufacturing environments proximate to where

the product contamination was observed. At the time of submission, the relationships of the organisms were unknown to the reference laboratory, and the sponsor was only able to determine that the isolates relevant to the contamination investigation were all coagulase-negative *Staphylococcus* spp. The submission request was to identify and subtype, potentially relating the contaminant isolate, to one or more of the environmental strains.

Because a large number of environmental isolates are *Staphylococcus* spp., recovery of one of these closely related organisms (e.g., *S. capitis* ssp. capitis, *S. capitis* ssp. urealyticus, *S. caprae*, *S. epidermidis,* and *S. saccharolyticus*) from a product may pose a challenge during bioburden root cause investigations. Accurate classification, not only at taxonomic but also strain levels, is crucial to enable definitive matching of a contaminant to a source strain recovered from the manufacturing environment.

Ribosomal Gene Sequencing

For this investigation, the 16S ribosomal rRNA gene from each bacterial isolate was sequenced, and relatedness of the DNA sequences reported (see Table 9.1). From the results presented, as obtained from a proprietary reference sequence database, it is clear that all the organisms submitted were *Staphylococcus* spp. The close genetic relatedness of the isolated organisms, however, made it impossible to ascribe species and taxonomic groupings (e.g., to differentiate *S. capitis* from *S. caprae*) based on genetic sequencing results alone. Additionally, whereas the contaminant (isolate A) had a DNA fingerprint profile similar to that of isolate B, the minor variations seen in the reported genetic distances from the reference strains could indicate that these two isolates, though very closely related, were not of the same strain and, thus, the source of environmental isolate B may not have been the cause of the overt contamination.

Phenotypic Analysis

In order to further investigate this contamination event, a typical phenotypic analysis of the microbial isolates was performed, with observation of colonial morphologies on various types of media and subsequent Gram-staining reactions. The results of these phenotypic observations are also presented in Table 9.1. From this analysis alone, it was clear, a contamination source could not be determined. Moreover, isolates A and B yielded different colonial morphologies although isolate A had a DNA matching profile similar to that of isolate B based on 16S rRNA gene sequence analysis. Additional testing was required using appropriate biochemical tests for Gram-positive bacteria, because conventional microbiological assays evaluating metabolic pathways and biochemical utilization patterns would perhaps provide additional data that could be helpful in the investigation. In the case of conventional methods, however, the ever-increasing number of new species being described makes this approach challenging—even for laboratories specialized in the archaic and sometimes arcane methods prescribed for conventional microbiology. Most of the phenotypic reactions used to differentiate the species of *Staphylococcus* are often either variable or insufficient to enable correct identification. In this particular case, the phenotypic reac-

TABLE 9.1
Application of Polyphasic Methods for Identification

	Isolate A (Product Contaminant)		Isolate B (Environmental)		Isolate C (Environmental)		Isolate D (Environmental)		Isolate E (Environmental)	
Test Samples	**Genus/Species**	**Genetic Distance**	**Genus/Species**	**Genetic Distance**	**Genus/Species**	**Genetic Distance**	**Genus/Species**	**Genetic Distance**	**Genus/Species**	**Genetic Distance**
DNA Sequencing results	*S. caprae*	0.0015	*S. capitis*	0.0000	*S. caprae*	0.0062	*S. caprae*	0.0024	*S. caprae*	0.0181
	S. capitis	0.0029	*S. caprae*	0.0007	*S. capitis*	0.0074	*S. capitis*	0.0046	*S. epidermidis*	0.0188
	S. saccharolyticus	0.0057	*S. saccharolyticus*	0.0030	*S. saccharolyticus*	0.0104	*S. saccharolyticus*	0.0047	*S. capitis*	0.0198
	S. epidermidis	0.0072	*S. epidermidis*	0.0080	*S. epidermidis*	0.0137	*S. epidermidis*	0.0108	*S. saccharolyticus*	0.0224
	S. aureus	0.0256	*S. aureus*	0.0220	*S. aureus*	0.0286	*S. aureus*	0.0275	*S. hominis*	0.0295
	Character/Test	**Result**	**Character/Test**	**Result**	**Character/Test**	**Result**	**Character/Test**	**Result**	**Character/Test**	**Result**
Phenotypic observations	Colony color	**Gray**	Colony color	**White**	Colony color	**White**	Colony color	**White**	Colony color	**White**
	Gram stain	+	Gram stain	+	Gram stain	+	Gram stain	+	Gram stain	+
	Shape	**Cocci**	Shape	**Cocci**	Shape	**Cocci**	Shape	**Cocci**	Shape	**Cocci**
	F or O	**F**	F or O	**F**	F or O	**F**	F or O	**F**	F or O	**F**
	Catalase	+	Catalase	+	Catalase	+	Catalase	+	Catalase	+
	Oxidase	–	Oxidase	–	Oxidase	–	Oxidase	–	Oxidase	–
Phenotypic reactions	Urea	+	Urea	(+)	Urea	–	Urea	–	Urea	–
	Trehalose	+	Trehalose	+	Trehalose	–	Trehalose	+	Trehalose	–
	Mannitol	+	Mannitol	+	Mannitol	+	Mannitol	+	Mannitol	–
	Lactose	+	Lactose	+	Lactose	–	Lactose	+	Lactose	–
	Sucrose	–	Sucrose	–	Sucrose	+	Sucrose	+	Sucrose	+
RMM ID[a]	*S. simulans*	80%	*S. haemolyticus*	95%	*S. capitis*	80%	*S. haemolyticus*	92%	*Corynebacterium xerosis*	67%
Final ID	*S. caprae* ssp. urealyticus		*S. caprae* ssp. urealyticus		*S. capitis* ssp. capitis		*Staphylococcus caprae*		*S. capitis* ssp. capitis	

[a] Percentages represent confidence levels of the RMM identification system.

Note: C = cocci; F = fermentative; O = oxidative; (+) = delayed reaction.

Source: Contributed by Molecular Epidemiology, Inc.

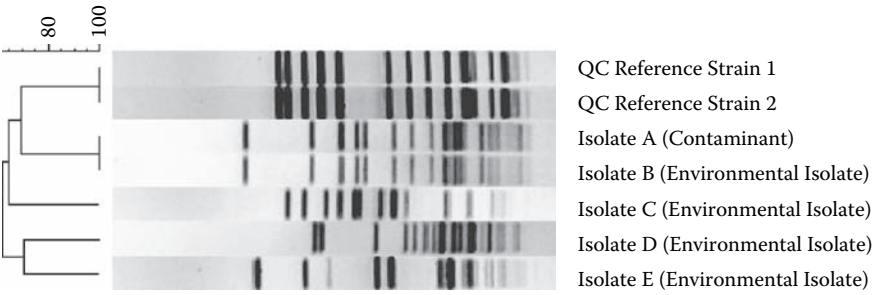


FIGURE 9.23 Genetic subtyping of *Staphylococcus* strains by pulsed field gel electrophoresis (PFGE). (Courtesy of Molecular Epidemiology, Inc., www.molecularepi.com. With permission.)

tions used to differentiate the species of interest (*S. caprae, S. capitis, S. epidermidis,* and *S. saccharolyticus*) were very few and limited, and the reported results were not corroborated in each case by the DNA sequence data. However, consistencies with published phenotypic results were observed, and upon examining a specific subset of the biochemical reaction results, a pattern became clear that, in turn, corroborated results from genetic subtyping analysis (see Figure 9.23). Indeed, identification of *Staphylococci* spp. by conventional phenotypic and RMM means can be a daunting task as evidenced by the presumptive *Corynebacterium* spp. identification result for one of the submitted isolates. Therefore, it is not surprising that RMM, which rely on phenotypic profiling, may fall short in correctly ascribing a taxonomic status to an unknown organism.

Genetic Subtyping—PFGE

The last phase in the polyphasic approach to identification of the bacterial isolates involved application of genetic subtyping methodologies. The use of techniques such as ribotyping or enzymatic cleavage of total genomic DNA, followed by pulsed-field gel electrophoresis of total genomic DNA (PFGE), provides reproducible patterns unique to clonal isolates (see Figure 9.23). These methods are used for very sensitive evaluation of individual strains of a species to determine if they are genomically related. Unfortunately, as powerful an analytical tool as genetic subtyping may be for matching genomic patterns, it is generally not adequate for identification, unless the clonal pattern and taxonomic identity of the organism has already been determined and submitted to the database. If a new isolate, unknown to the existing database, is submitted for evaluation, there will be no match and no adequate way to definitively identify the organism, percentage matches notwithstanding. As evidenced by the different patterns exhibited by the *S. capitis* ssp. capitis strains shown in Figure 9.23 (isolate C and isolate E), there can be a number of different patterns for the same species (in this case, even subspecies) with no definitive match.

Results and Reporting

It was evident that, individually, the different identification approaches were unable to provide results for an unequivocal conclusion as to the correct taxonomic place-

ment of the unknown isolates. However, the polyphasic approach, which combines three different technologies, enabled us to arrive at the following supportive and differential information:

1. Results obtained from 16S rRNA gene sequencing supported the genus *Staphylococcus* for the isolates—in particular, the closely related species *capitis, caprae, saccharolyticus,* and *epidermidis*.
2. Phenotypic observations (including Gram-stain) and biochemical reactions supported the genus *Staphylococcus*. By relying on the genetic analysis to confirm that the correct genus was indeed *Staphylococcus*, and noting that the phenotypic results did not refute that genus, the remaining time and resources could then be spent on separating the various species.
3. Specific and selected reactions were used to differentiate the closely related species. In this case study, by focusing on the differential reactions of urea hydrolysis, and utilizing lactose, mannitol, sucrose, and trehalose, the two distinct species and subtypes were differentiated.
4. Genetic subtyping data supported the premise that a close genetic relationship existed, and the data also provided sufficient discrimination to distinguish which isolates were related (that is, matching product contamination to environmental isolate), and which were different.
5. Specifically, the 16s rRNA gene sequence data confirmed that the phenotypic conclusions not previously mentioned (*Corynebacterium* spp. or *Staphylococcus* spp.) were incorrect or nonspecific. However, the phenotypic data provided the additional separation data required for assigning specific taxon based on published tables (see Table 9.1).
6. Genetic subtyping data (PFGE profiles) confirmed the discrimination provided by the phenotypic data; it demonstrated the conformity of two strains as clones and the unique profiles of the other strains.

In this case study, using polyphasic methods, we were able to differentiate isolate D as *Staphylococcus caprae*, distinctively separate from the other closely related species. Further, we demonstrated that isolates C and E were both *S. capitis* ssp. capitis, but they were not closely related strains. Even though they were identified at the subspecies level, we could prove that there were sufficient differences (based on banding patterns) between them to accord each a unique strain status. Finally, the product contaminant (isolate A) and isolate B both were identified as *S. caprae* ssp. urealyticus, and they had identical DNA fingerprint patterns. This perfect match in taxonomic identity not only in subspecies but also in genetic subtyping provided evidence of the exact relationship between the two isolates and, hence, association with the point in the manufacturing process where environmental isolate B was recovered. The unambiguous identification of the contaminant strain provided insight for effective corrective and preventative action (CAPA) for eradication of the contamination source in the process stream.

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10 Biofilms

Microorganisms have been forming biofilms for millions of years. However, the understanding and control of biofilms is a relatively new area of study in microbiology. In fact, only as recent as ten years ago did researchers recognize the distinct phenotype of a biofilm as the predominant type of bacterial life. In this chapter, the author will address the basics of biofilm structure and the impact of biofilms in equipment contamination and pharmaceutical production. The author will also address testing of sanitizer efficacy using biofilm cells grown in a laboratory setting.

BIOFILM DEFINITION

In 1978, biofilms were first defined as being unstructured aggregations of bacterial cells because scientists did not have access to today's technologies. Primarily, they were using flawed electron microscopic techniques that require complete dehydration of the highly hydrated biofilm mass (95% water), as well as distorted/out-of-focus observations made using light microscopy. This caused scientists to misjudge the complexity of these well-organized and elaborate microbial communities [1]. Today, we know that biofilms are complex and three-dimensional microbial sessile communities of cells embedded in a matrix of extra polymeric substances (EPS) and irreversibly attached to a substrate, interface, or to each other [2]. In addition, researchers have come to realize that biofilms are universal; biofilm is found to be a preferred way of life for bacteria because it represents a survival strategy as a multilayer defense mechanism against environmental stresses. Figure 10.1 illustrates a biofilm composed of *Staphylococcus aureus* bacteria attached to an indwelling catheter. The EPS matrix secreted by the organisms, which helps protect and keep the microbial community together, is clearly visible.

BIOFILM STRUCTURE

A native biofilm, which has a microcolony as its basic structural unit, contains about 15% microbial cells and 85% EPS, the latter composed of polysaccharides, proteins, other polymers, and water. The EPS matrix forms "towers" and "mushrooms," shapes seen in Figure 10.2. Within these towers, water channels are formed interspersed between the sessile microbial cells where nutrients, oxygen, and waste flow through. The chemical composition, physical properties, hydrophobicity, and solubility of the EPS matrix vary with the microorganisms, and age of biofilm, and also within the

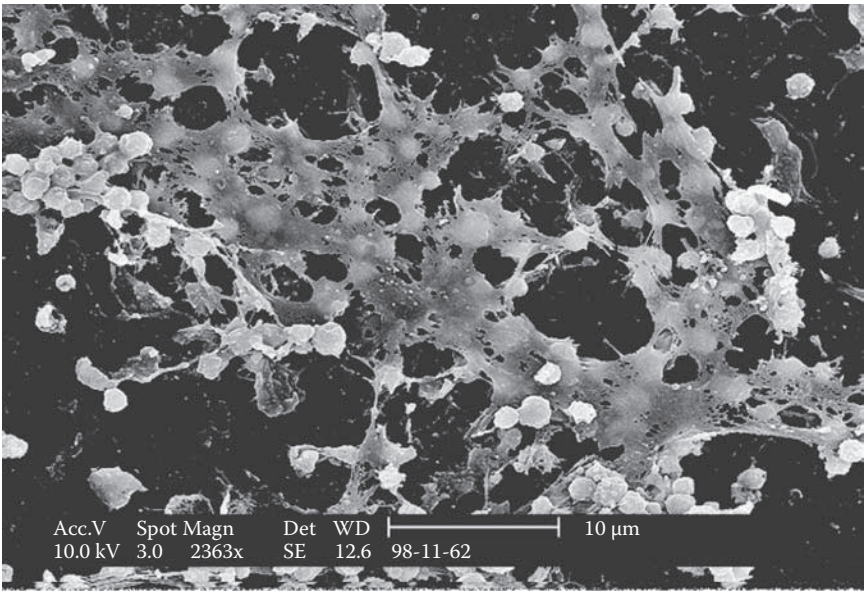

FIGURE 10.1 Scanning electron micrograph depicting a *Staphylococcus aureus* biofilm found on the luminal surface of an indwelling catheter. (From Public Health Image Library, Center for Disease Control and Prevention, U.S. Department of Health and Human Services. With permission.)

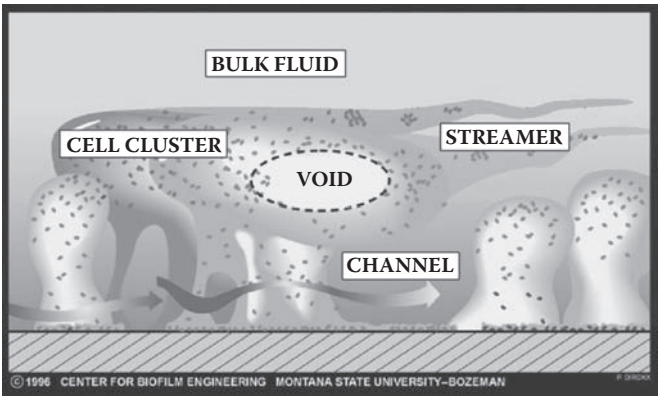

FIGURE 10.2 Conceptual illustration of biofilm structure showing bacterial clusters, streamers, and water channels. (Courtesy of P. Dirckx, Montana State University Center for Biofilm Engineering, Bozeman. With permission.)

biofilm structure itself. For example, most Gram-negative organisms secrete polysaccharides that are neutral or polyanionic, whereas Gram-positive bacteria secrete EPS that are quite different chemically and tend to be cationic [3]. Biofilms also have aerobic and anaerobic portions. Even in a perfectly aerobic environment (e.g., a very thin biofilm), the head of the mushroom is anaerobic. Often, noncellular materials, such as mineral crystals and corrosion particles, are found within the biofilm matrix.

Biofilms are not rigid structures; they have an inherent viscoelasticity resembling that of rubber. If a biofilm is formed in a slow-flow or stagnant environment, it will be soft and it will come off very easily. However, when formed in a high-flow environment, biofilms are tough and rubbery. If one tries to remove the biofilm with a pressure surge, the surviving cells will form a biofilm that will get more rubbery; and if this process continues, eventually one will end up with a biofilm that is as hard as dental plaque.

THE BIOLOGY OF BIOFILMS

A biofilm is often initiated by microcolonies from one type of organism. However, biofilms (mainly environmental) quickly become heterogeneous as mixed cultures of bacteria, as well as fungi, algae, and protozoa, join the established structure and become intermixed. In fact, within a biofilm, different types of microorganisms can coexist and form stable communities, whereas others may compete for the environment, resulting in dominance by one species over another. Studies demonstrated that biofilms formed by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are stable and able to thrive quite well in aqueous environments. *P. aeruginosa* is the first organism to quickly colonize the surface, followed by *K. pneumoniae*, which attaches to the *P. aeruginosa* biofilm and outcompetes for the aerobic surface layers of the biofilm [4].

Biofilm Formation

Biofilms can form on nearly any type of substratum. The process of biofilm formation begins when planktonic bacteria encounter a surface and turn on genes that will allow them to become sessile and adhere to the surface. There are three main stages in biofilm formation as illustrated in Figure 10.3. Stage 1 comprises the initial adhesion to a surface; Stage 2 involves cell growth or reproduction and production of EPS, followed by an irreversible adhesion to the surface. The trapped biofilm bacteria form a community that controls the structural complexity of the biofilm. Stage 3 is when detachment of sessile microcolonies occurs, and these cells travel to form new biofilms on other locations.

Quorum Sensing

Quorum sensing plays a key role in the initial stages of biofilm formation and in biofilm dispersion. This phenomenon is a type of cell-to-cell signaling mechanism that enables a bacterium to regulate gene expression in response to cell population density, and facilitates an organism's adaptation to changing environmental conditions. This type of intracellular communication, which occurs both within and

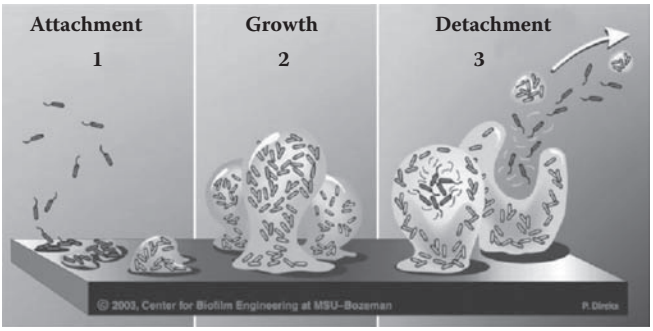

FIGURE 10.3 Illustration of stages of biofilm formation. (Photo courtesy of P. Dirckx, Montana State University Center for Biofilm Engineering, Bozeman. With permission.)

between species, was first described in two marine bioluminescent bacteria, *Vibrio fischeri* and *Vibrio harveyi*, in which luminescence is expressed at high cell densities [5]. Quorum sensing depends on the production of diffusible signal molecules called *autoinducers* (AI) or *pheromones*. Once AI molecules reach a high concentration, they start to interact with regulatory proteins that modulate gene expression. Besides bioluminescence, cell adhesion, and cell detachment, a variety of other physiological processes is regulated by quorum sensing, and those include swarming, motility, sporulation, conjugation, and production of virulent molecules [6].

Gram-negative bacteria have two types of autoinducers, AI-1 and AI-2. The AI-1 molecules are *N*-acyl-homoserine lactones (AHL) and the AI-2 molecule is a furanosyl borate diester. The AI-1 regulatory system is comprised of two structural genes: *luxI* that encodes the AI-1 synthase and *luxR* that encodes the AI-1 response regulator. The gene responsible for AI-2 production is *luxS*, which is highly conserved across many microbial species. The ability of AI-2 to regulate gene expression in many types of bacterial species indicates a possible role in interspecies communication as opposed to intraspecies communication that is typical of AI-1 [7]. Quorum sensing in Gram-positive bacteria, although identical in purpose to the one used by Gram-negative bacteria, differs in the density-dependent expression of target genes, the signaling molecules, the mechanism of their synthesis, and the secretion and detection apparatus. Gram-positive bacteria use processed oligopeptides (secreted peptides) as AIs. Typically, the peptide signal molecule is exported by a dedicated ATP-binding cassette (ABC) transporter, posttranslationally modified, and finally sensed by other cells via membrane-located receptors that are part of a two-component regulatory system [8].

Cell Adhesion

The initial bacterial cell adhesion to surfaces is a process governed by long-range forces, primarily van der Waals and electrostatic interactions. This initial stage of biofilm formation is dependent on several factors, including bacterial cell properties (e.g., hydrophobicity); the nature, type, shape, and physicochemical properties of the substratum as well as the chemical composition, hydrodynamics, and flow characteristics of the liquid environment. In general, an increase in nutrient concentration

leads to increased rate of attachment. Also, motile bacteria seem to attach in greater numbers as compared to nonmotile strains.

Smooth versus Rough Surfaces

It is widely accepted in the pharmaceutical industry that smooth surfaces, such as electropolished stainless steel, offer resistance to bacterial cell adhesion. However, recent studies may call such a claim into question in light of the scientific evidence that biological surfaces that are rougher actually seem to be better at preventing bacterial adhesion [9]. Therefore, it appears that surface roughness may not, after all, have a great impact on initial cell adhesion. However, rough surfaces and surfaces with imperfections should be avoided as they are harder to clean and can create pockets where bacteria can collect, thus leading to potential microbial colonization and biofilm development.

Hydrophobic versus Hydrophilic Surfaces

Hydrophobicity of microbes plays a role in microbial adhesion to substrata. Microorganisms can be hydrophilic, hydrophobic, or both, because changes in gene expression, such as production of flagella and fimbrae, can change the hydrophobicity of microbes. For example, streptococci and lactobacilli can be either hydrophilic or hydrophobic, and although nearly all staphylococci appear to be hydrophilic, some hydrophobic strains, such as *Staphylococcus epidermidis* 3294, exist [10]. In general, hydrophobic organisms tend to aggregate in aqueous suspensions and are often repelled by hydrophilic surfaces such as glass and metals. In contrast, hydrophilic organisms prefer the aqueous phase and are often repelled by hydrophobic surfaces such as Teflon®, polyethylene, polystyrene, etc. In addition, the production of mucolic acids by Gram-positive bacteria appears to help with attachment to hydrophobic surfaces, whereas the EPS and the lipopolysaccharide (LPS) layer of Gram-negative rods seem to help with attachment to hydrophilic materials.

Electrostatic Charge Properties

Electrostatic charges, as indicated earlier, play a role in bacterial cell adhesion. Negatively charged surfaces, such as Teflon, repel microbial cells that are negatively charged, whereas positively charged surfaces repel microbial cells that are positively charged. Because typical microbial cell surfaces are negatively charged, one could make the general assumption that Teflon can resist biofilm formation. However, recent studies have identified positively charged organisms that adhere tenaciously to negatively charged substrata [11]. Cell charges also change depending on expression of cell-surface proteins and, therefore, even in a pure-culture biofilm, there may be a distribution of cell charges that can impact cell adhesion to various types of surfaces.

Low-Shear versus High-Shear Environments

Although bacterial cell adhesion was believed to be a phenomenon associated primarily with low-shear/stagnant environments, recent studies indicate that bacteria can attach to surfaces and form biofilms in high-shear/turbulent flow environments

exceeding Reynold's numbers of 5000 [12]. The Reynold's number (Re) is a dimensionless value used in fluid mechanics to indicate whether a fluid flow is laminar or turbulent. Re values below 2000 represent *laminar flow*, those between 2000 and 4000 represent a *transition phase*, and Re values above 4000 denote *turbulent flow*. Bacterial attachment to surfaces under high-shear conditions is possible due to the fact that, even under the most turbulent conditions, there is always a stagnant boundary layer between the fluid and the solid surface where the bacterial cells are impinged onto, thus allowing biofilms to form.

Based on all the recent findings on bacterial cell adhesion, it is now clear that general assumptions concerning the physicochemical properties of microbial cells and the likelihood of a material to resist bacterial adhesion cannot be made. The traditional concepts that initial cell adhesion is more prominent on surfaces that are rougher and more hydrophobic and when under low-shear conditions now appear to have been based on data generated using environmental isolates that had lost their "wild" phenotypes during laboratory experiments and did not truly behave as their wild biofilm counterparts.

Biofilm Dispersion

Biofilm cells can be dispersed by three main processes: shedding, detachment, and by physical forces. *Shedding* occurs when daughter cells separate from an actively growing biofilm, a process that is still not fully understood. *Detachment* appears to be species specific, and is believed to occur as a result of low-nutrient levels or quorum sensing. Dispersion by physical forces include *continuous erosion or shearing* of small and top layers of the biofilm; *sloughing,* causing a rapid removal of a large portion of the cell mass; and *abrasion*, which occurs due to collision of particles from the fluid medium onto the biofilm. Sloughing seems to be more random than erosion and is thought to occur when there is depletion of nutrients or oxygen in the biofilm. Abrasion seems to occur more often in environments containing large numbers of particulates.

The ability to sense and migrate to optimum environmental conditions for growth confers on microorganisms a survival advantage and a means of competition. However, in a pharmaceutical manufacturing environment, dispersion of biofilm cells can have detrimental effects on a process unit operation, a piece of equipment, or water system. Dispersed cells often maintain their biofilm phenotype, including antimicrobial resistance and the ability to attach to new surfaces, thus posing a risk for systemic microbial colonization and potential product contamination.

Biofilm Resistance and Phenotypes

Microorganisms present in a biofilm exhibit an altered phenotype with respect to growth rate and gene transcription, and behave quite differently from their planktonic counterparts. The difference is actually greater when comparing the phenotype of a spore and a vegetative cell; it takes 65 genes for a bacterium to sporulate as compared to 85 genes for a planktonic cell to turn into a biofilm cell [13]. In a way, the bacterium becomes a different organism: it will express different proteins and become much more resistant to adverse environmental conditions, environmen-

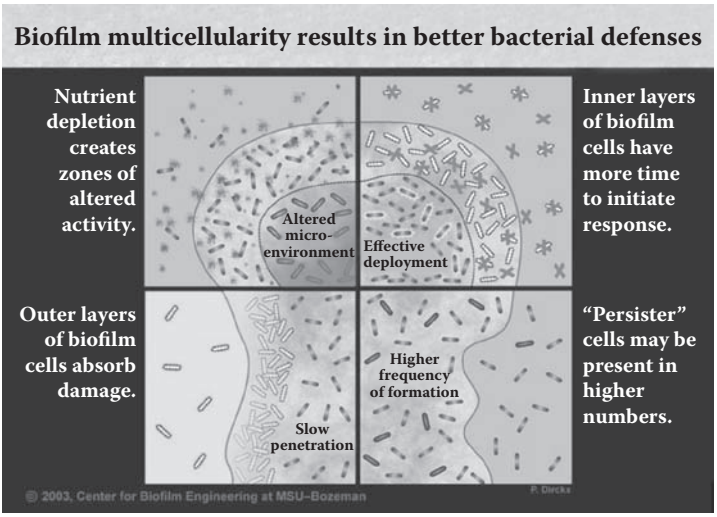

FIGURE 10.4 Illustration of the multicellular defense mechanism in biofilms against chemical and environmental stresses (Photo courtesy of P. Dirckx, Montana State University Center for Biofilm Engineering, Bozeman. With permission.)

tal bacteriophages, and phagocytic amoebae. In fact, biofilms are considered to be 100% phage proof.

Biofilms are also resistant to many chemicals that are lethal to planktonic cells. A study to evaluate biofilm removal by various chemical treatments indicated that multiple interactive forces contribute to biofilm cohesion, making it difficult for chemicals that are effective against planktonic cells to destroy biofilm organisms [14]. The unique antimicrobial resistance of biofilm cells is an important distinction between bacterial communities that attach to surfaces but that do not assume the biofilm phenotype. These "nonbiofilm" populations, although sessile, behave similar to planktonic cells and are therefore much more susceptible to adverse conditions.

Other factors that affect biofilm resistance include a multilayer defense system that includes slow penetration of chemicals through the EPS matrix, an altered microenvironment in the center of the biofilm, and the presence of putative *persister* cells that appear to be in a dormant metabolic state [15]. Figure 10.4 illustrates how the multicellular characteristics of biofilms provide protection against chemicals and environmental stresses.

Understanding the physical and biochemical characteristics of biofilms is very important so that scientifically sound test protocols to challenge antimicrobial efficacy of sanitizers and disinfectants are created. One of the critical design aspects of such studies is the preparation of microbial suspensions to ensure that biofilm cells maintain their wild phenotype and metabolic state during laboratory testing. Indeed, the standardization and storage of microbial suspensions are critical because as soon as a portion of a biofilm is removed from the environment and cultured in the laboratory (typically within one passage from original culture), the microorganisms will loose their biofilm phenotype and increased resistance to chemicals and environmental stresses and will go back to a planktonic phenotype. Freezing the prepared

microbial suspensions after the first subculture in a low-nutrient medium is often an adequate preventive procedure.

PHARMACEUTICAL PRODUCTION EQUIPMENT AND MATERIALS PRONE TO BIOFILM FORMATION

The equipment used in pharmaceutical and biotech manufacturing should meet good engineering design and principles. The American Society of Mechanical Engineers (ASME) sets the standards for bioprocessing equipment; ASME BPE-2002 provides the requirements applicable to the design of manufacturing equipment, including aspects that relate to sterility and cleanability, dimensions and tolerances, surface finish, material joining, and seals. All product contact equipment (including valves and pumps) should be of sanitary design as defined by the 3-A Sanitary Standards (www.3-A.org). The following listed requirements represent the industry's accepted minimum design principles for production equipment, and are critical to ensure microbial control and prevention of biofilm formation.

- *Cleanability*: All product contact surfaces must be accessible to the cleaning solutions. Internal horizontal product contact surfaces must be minimized. Equipment must be drainable and free of areas where liquid or soil may collect. Equipment must be free of areas of low flow and low velocity; fasteners or threads must not be exposed to the process, steam, or cleaning fluids.
- *Sterility*: Steam sterilizable equipment parts must withstand continuous flow of saturated steam at a minimum temperature of 130°C.
- *Surface finishes*: Typically, materials such as 316 or 316L stainless steel or higher-grade materials should be used as surface. When nonmetallic surfaces are used (e.g., plastics and elastomers), the company must demonstrate that the material complies with the FDA 21CFR 177 and the USP Class VI standards. Transparent materials such as glass and borosilicate must be scratch proof, inert, nontoxic, and rated for the applicable pressure and temperature of vessels where used. Some applications in which glass is used besides glassware (more commonly Pyrex®) are light and sight opening into vessels and chromatography columns.
- *O-rings, seals, and gaskets*: These materials when used in piping and tubing must be of the correct size, flush with the interior surface of the pipeline or the equipment, and compatible with clean-in-place (CIP) cycles.
- *Connections and fittings*: The equipment design should minimize the number of connections. Design for connections and fittings must be sanitary, ensuring that when pressure builds up on each side of the gasket, product will not get stored in crevices that might exist in joints otherwise watertight. Connections must be able to be cleaned in place, fittings must not have any hard-to-clean crevices around the gasketed joint; seals must not be deformed due to an increase in temperature or pressure, and all product contact fittings must be self-draining. No dead legs should be present in the system; a dead leg is defined as an area in a piping system where liquid can become stagnant and not be exchanged during flushing.

In general, most of the equipment used in pharmaceutical production do meet the ASME and 3-A standards. Systems and equipment that become contaminated with microorganisms either have undesirable design features, are difficult to clean, or are improperly maintained. A detailed discussion on the types of systems and equipment used in biopharmaceutical manufacturing that are prone to biofilm formation is presented in the following sections.

Water Systems

A well-designed water system that has been validated and maintained via appropriate sanitization practices is able to produce water that meets microbial and chemical quality attributes defined by the regulatory agencies and the compendia. Pharmaceutical grade waters are not expected to be sterile (unless defined as such and manufactured in a manner to ensure sterility). As discussed in Chapter 4, purified water has a recommended bioburden limit of 100 CFU/mL, and water for injection (WFI) has a recommended bioburden limit of 10 CFU/100 mL.

The FDA and the International Society for Pharmaceutical Engineering (ISPE) have published guidance documents for the construction, maintenance, and inspection of high-purity water systems. There are also many articles and books written on water for pharmaceutical use to help manufacturers with their water system design needs and decisions. Important design features for prevention of biofilm formation in water systems are the material of construction, temperature of the system, and water flow. Stainless steel piping should be the preferred material due to ease of cleanability and suitability for heat sanitization. Companies that choose polypropylene (PP) or polyvinyl chloride (PVC) piping to reduce initial installation costs will most likely incur high maintenance costs later; these types of materials cannot be heat sanitized or ozonated, thus leading to a greater potential for biofilm formation.

WFI systems that are maintained circulating with a turbulent flow and at a high temperature (65–80°C) are deemed self-sanitizing. Purified water systems that are maintained circulating with a turbulent flow and at ambient temperature (25±5°C) or cold WFI systems (0–5°C) are typically steam sanitized once a week, with usepoints heat sanitized daily. These practices are often effective in preventing biofilm formation. Maintaining a circulating water system is critical because a one-way water system is basically a dead leg.

In pharmaceutical manufacturing, the risk of systemic microbial contamination in a water system is low due to requirements for extensive validation work and routine monitoring. Feedwater is also monitored frequently to evaluate seasonal variations in microbial population that could adversely affect the operation of the water system. In cases where bioburden is detected in a grab water sample, it is often associated with port contamination due to improper flushing/sanitization or personnel related to adventitious contamination during sampling.

Pharmaceutical water systems may become contaminated due to a failure in the maintenance or operating procedures designed to prevent introduction of microorganisms into the system. For example, nonsterile air that remains in a pipe, valve, or hose after drainage may be introduced into the system inadvertently. Pretreatment of feedwater is recommended by most manufacturers of distillation equipment to

prevent unacceptable levels of microbes and endotoxins in the distillate (e.g., WFI). Carbon beds used for pretreatment of feedwater can become a breeding ground for biofilms, and these units must be heat sanitized as backwashing does not work and can exacerbate the problem.

Perforated heat exchangers can also lead to contamination of a water system. The FDA technical guide, Heat Exchangers to Avoid Contamination, discusses the design and potential problems associated with heat exchangers [16]. The guide references two main methods for preventing contamination by leakage: (a) provide gauges to constantly monitor pressure differentials in order to ensure that the higher pressure is always on the clean fluid side and (b) utilize the double-tube sheet type heat exchanger. Also, as a preventive measure, the FDA recommends that heat exchangers not be drained of the cooling water when not in use to prevent pinholes from being formed in the tubing after they are drained as a result of corrosion of the stainless steel tubes in the presence of moisture and air.

Although the presence of dead legs is not a desired design feature, there are systems (and equipment) that have dead legs, which can lead to potential biofilm formation. Dead legs in a hot-water system may be less of a concern. However, in cooler systems, any dead leg will have the potential for microbial colonization. Microbial contamination can also occur if pumps are not continuously in operation, resulting in a static reservoir area where water will become stagnant. In such cases, a company may need to install a drain from the low point in the pump housing.

In many small biotech companies, reverse osmosis (RO) and ultra filtration (UF) systems are used to produce high-purity water—some companies even have small wall-mounted units. In large companies, RO systems are used as pretreatment for highly purified waters. However, RO systems, if not of sanitary design, are prone to microbial contamination that often becomes established in the membrane filters and in the ball valves; the center of the valve can collect water when the valve is closed, and the stagnant water can harbor microorganisms and provide a starting point for the development of a biofilm. With the recognition of the dangers of potential biofilm formation in RO units, filter manufacturers recommend installing at least two units in series, and some manufacturers have installed heat exchangers immediately after the RO filters to heat the water to 75–80°C in an attempt to minimize microbial contamination. In addition, an ultraviolet (UV) light is often installed in the system downstream from the RO units to aid in the control of microbial proliferation.

Although ozone and UV light have been used to control microbial contamination in water systems, both methods have pros and cons. Ozone systems can be relatively inexpensive, but for optimum effectiveness, the dissolved residual ozone must remain in the system, thus presenting safety concerns not only for employees but also for drugs formulated with the water. Another concern with using ozone is that ozonating the incoming water breaks up many types of nutrients that otherwise would not be available for uptake by microorganisms. Therefore, for some water systems, ozone makes nutrients available to bacteria, and a biofilm bloom can develop immediately after the ozonater. The effectiveness of UV lights for control of microbial contamination is limited and dependent on where the unit is located, and whether the UV light is on continuously or just turned on when water is needed. UV light penetrates biofilms poorly. Much of the radiation gets trapped in the EPS matrix, so the sessile cells are

protected from and resistant to the radiation. UV lights must also be properly maintained to work as intended. The glass sleeves around the bulbs must be kept clean, otherwise their effectiveness is decreased. Therefore, relying solely on UV light for microbial control is not recommended. For optimum bioburden control in water systems, experts recommend pairing UV lights with microbial retention filters.

Production Equipment

Microbial contamination of pharmaceutical or biopharmaceutical equipment occurs primarily with equipment and materials that do not meet sanitary design standards, or that cannot handle steam sterilization or autoclaving and, therefore, must be chemically sanitized. The PDA Survey on Aseptic Processing—2001 [17] identified aseptic connections as one of the most common reasons for introduction of microbial contamination into aseptic process streams, which can eventually proliferate into biofilms. Usually, aseptic connections are made using Tri-Clover fittings and connectors that are autoclaved prior to use. However, the integrity of the aseptic connections depends on the cleanliness of the manufacturing area, microbial barriers (e.g., personnel gowning), and training and competency of operators in aseptic techniques. Other areas of concern include nondisposable filters, sampling or transfer hoses, joints, valves, seals, and gaskets. Often, biofilm is formed behind seals and gaskets that are not of proper size or type for the equipment in which they are installed. In addition, equipment surfaces that have become corroded will be more prone to biofilm development due to soil and bacteria collection in pitted areas and cracks.

In biopharmaceutical manufacturing, most microbial contamination events occur in downstream processing (e.g., purification) because most, if not all, equipment and materials used in upstream manufacturing (e.g., fermentation and cell culture) can be either autoclaved or steam sterilized. Areas of concern in upstream manufacturing are heat exchangers that could develop leaks as well as sensor probes that cannot be steamed-in-place and may become an entry route for microbial contamination into the bioreactor. In downstream processing, the chance for equipment biofouling is greater because many environmental microorganisms present in sessile microbial communities become resistant to the typical chemical sanitizers used for equipment cleaning and storage. Based on the author's experience, which is supported by industry experts, ultra filtration/diafiltration (UF/DF) and chromatography systems (and associated process hoses) are the two downstream unit operations most prone to microbial contamination. A detailed discussion on these two systems is presented in the following sections.

Ultrafiltration/Diafiltration (UF/DF) Systems

UF and DF are filtration systems (membrane filter and module) used in biotechnology for clarifying, concentrating, and purifying proteins. Filtration can be accomplished via normal flow filtration (NFF) also called *dead-end filtration* and tangential flow filtration (TFF) also known as *cross-flow filtration*. In an NFF procedure, the fluid medium is pumped directly toward the membrane under an applied pressure. Particles that are smaller than the membrane pore size pass through the filter to the downstream

side whereas larger particles accumulate at the membrane surface. In a TFF procedure, the fluid medium is pumped tangentially along the surface of the membrane, and the applied pressure forces particles that are smaller than the membrane pores to pass through the filter and into the filtrate side. The main difference between NFF and TFF is that in the latter, particles too large to pass through the membrane pores do not accumulate on the filter membrane and are instead swept along by the tangential flow.

UF is a form of TFF widely used to separate proteins from buffer components for buffer exchange, desalting, or concentration. DF is a technique often applied in combination with a UF step designed to improve either product yield or purity. With the use of UF membranes, DF is able to completely remove (replace) or lower the concentration of salts and solvents from solutions. There are many reasons why a UF/DF system is prone to biofilm formation and they include

- *System design*: UF/DF units often contain dead legs, which can result in small volumes of solution being held up in the pipes and modules, thus creating pools of liquid with potential for biofilm formation. TFF modules vary in design, and some have screens inserted into the feed and filtrate channels in spiral-wound and flat-plate modules to increase turbulence in the channels and reduce concentration polarization (this is not an option with hollow-fiber modules). These screens can be an area of concern when it comes to sanitization. TFF systems require the use of multiple flexible hoses (for buffer and product transfer) that are very difficult to clean and sanitize, thus contributing to a high risk of biofilm development.
- *Filter membranes*: Some types of filters can provide a good environment for microbial colonization. Two of the most common materials used for UF membranes are regenerated cellulose and polyethersulfone (PES). Regenerated cellulose membranes are very hydrophilic, exhibiting superior cleanability (low biofouling) and ultralow protein adsorption. These membranes are compatible with organic solvents but are less tolerant to extreme pH, a characteristic that may affect the choice of sanitizing agent. PES membranes tend to adsorb proteins and other biological components, thus leading quite often to membrane fouling and lowered flux. The Biomax® (Millipore Corporation, www.millipore.com) is a type of PES membrane that has been hydrophilically modified to resist biofouling. Biomax membranes are highly stable and can withstand wide temperature and pH ranges. Millipore recommends the Biomax membrane for applications using harsh pH conditions, either during processing or during cleaning.
- *Process design*: UF/DF processes are run under sanitized but nonsterile conditions, and most UF/DF membranes do not have sterility claims from the manufacturer. Therefore, there is a greater risk for higher bioburden loads especially during extended processing times.

Chromatography Systems

Chromatography is widely used in bioprocessing for the isolation and purification of proteins, peptides, and other molecules from complex mixtures. Chromatography

columns and skids have certain intrinsic cleaning challenges making them prone to microbial contamination. For example:

1. Packed beds influence hydraulic dynamics within a column, making it difficult to achieve the high linear velocities necessary for CIP procedures.
2. Accessibility to column resin particles varies, creating sampling difficulties because the effectiveness of CIP is based on exposure.
3. Ligands may be sensitive to the cleaning agents.
4. Resin affinities may bind biomolecules that should be removed.
5. Chromatography skid parts such as O-rings, seals, bubble traps, valves, sensors, T-intersections, and dead legs (if present) can be difficult to clean.
6. Ancillary equipment such as pumps and hoses also add to difficulties during cleaning.

Although UF/DF and chromatography systems are more prone to microbial contamination as compared to other systems used in biopharmaceutical manufacturing, there are ways to minimize this risk. For example, it is critical to ensure that cleaning or sanitization cycles and storage solutions have been qualified, preferably using laboratory prepared biofilms. In addition, a company should set up a program to monitor the continued effectiveness of its equipment sanitization program. For UF/DF systems, the author recommends monitoring the bioburden of the retentate/filtrate material (in-process sample) to monitor the microbial quality of the UF/DF system over time or verify effectiveness of the cleaning procedure.

For chromatography systems, companies should test the column equilibration buffer eluate for bioburden content against established acceptable limits, a practice enforced by the regulatory agencies. Indeed, over the years various companies have received FDA observations for either not testing the bioload in the column eluate or for not evaluating the columns as potential sources of microbial contamination. The following are examples of regulatory citations on this topic:

- FDA 483 (BioQuality—Vol.10(10), October 2005: Cleaning validations conducted were inadequate in that they did not include testing for bioburden; there are no established bioburden specifications for equipment rinse samples, for example, UF/DF filters and purification column resins; no life span validations to support cleaned production equipment storage, for example: UF/DF filters and purification column resins.
- FDA 483 (BioQuality—Vol. 11(5), May 2006: No bioburden specification for inprocess materials (column eluates); purification columns not monitored for bioburden prior to use; deficient handling of out-of-specification results for purification column resin bioburden; no investigation, no quality impact on products manufactured; no preventative or corrective measures; same resins used in subsequent manufacturing of other product lots.
- FDA Consent Decree to Parkedale Pharmaceuticals, Inc., March 10, 2000: "While we acknowledge your promises of improvements in control of the environment in the … column room, you have not adequately addressed the … columns as sources of bioburden. For example, the … and the …

> are reused repeatedly and only changed between strains or if the flow rate decreases to a certain level. The sanitization of the … media and … has not been adequately validated and the number of uses of the … column has still not been established. You state in Parkedale's November 15, 1999 letter that the … column sanitization validation will be completed during the 1999/2000 Fluogen manufacturing season, and you acknowledge that formal qualification of the … filtration columns has not previously been completed. This response is unacceptable to FDA."

Chromatography column product eluates should also be tested for bioburden (in-process sample) prior to the filtration step that typically follows a chromatography unit operation. If bioburden samples are collected only from the product collection vessel post filtration, the company will not be able to effectively monitor the microbial quality of the product material leaving the column.

Miscellaneous Parts and Materials

In reality, any type of surface can develop biofilm in the presence of microorganisms and an aqueous environment. Although recent laboratory studies have indicated that material type may not influence the initial cell adhesion, when used in bioprocessing operations, some types of materials seem to be more prone to microbial contamination. Materials widely used in equipment parts in pharmaceutical manufacturing include stainless steel (316 and 316L), glass, acrylic, Teflon®, silicone, polypropylene (PP), polyvinylidine fluoride (PVDF), and ethylene propylene diene monomer (EPDM). Practical experience indicates that surfaces that are easily wetted and that are porous and/or have imperfections (not smooth) where liquid can collect are typically more prone to microbial colonization. If these types of surfaces are not cleaned in a timely manner or heat sanitized, biofilm may develop.

Over the years, industrial engineers have tried to create surfaces or treat existing surfaces with chemicals that would make them unsuitable for microbial cell adhesion. For example, it is common practice in the pharmaceutical and biotech industries to passivate stainless steel surfaces via exposure to a solution of nitric acid or of nitric acid plus oxidizing salts. This process restores the original corrosion-resistant surface by forming a thin transparent oxide film, and dissolves any embedded or smeared iron accumulated on the surface. Passivation is time consuming and expensive, and must be repeated at regular intervals (typically annually) to ensure that the iron in the material does not oxidize and also whenever additional weldings are performed. Even after undergoing passivation, stainless steel is wettable, which can enhance microbial adhesion and corrosion. However, given the fact that equipment, piping, and parts made out of stainless steel can be easily cleaned and steamed in place or autoclaved, biofilm development on stainless steel surfaces is less common.

Glass items are also wettable but can be steam sterilized or autoclaved, thus becoming less of a concern. Not all plastic and rubber parts, which are considered hydrophobic materials, can be autoclaved or steamed in place. Items that can withstand moist heat sterilization meet manufacturers' criteria for autoclaving (e.g., polypropylene containers marked "PP"). PP, silicone tubing, polypropylene copolymer (PPCO), polymethylpentene (PMP), Tefzel® ethylene tetrafluoroethylene (ETFE), Teflon tetrafluo-

TABLE 10.1
Removal of Biofilm in Dilute Sodium Hypochlorite

Material	*K. pneumonia* (% Removal)	*S. choleraisuis* (% Removal)	*E. coli* (% Removal)
Stainless steel (electropolished)	67	25	56
Polypropylene	67	75	75
Borosilicate glass	89	0	0
Silicone-coated glass	89	89	78
Polyvinylidene fluoride	89	89	89
Teflon® PFA	99	99	98

Source: Reported by the BioProcess Technical Institute, University of Minnesota.

roethylene-perfluoropropylene (FEP), and perfluoroalkoxy (PFA) can be autoclaved repeatedly at 121°C/15 psi, using a validated cycle. Polysulfone (PSF) and polycarbonate (PC) materials are autoclavable but can be weakened by repeated cycles, and may eventually fail under high-stress applications. Polystyrene (PS), PVC, nylon, acrylic, low-density polyethylene (LDPE), high-density polyethylene (HDPE), and polyurethane tubing are not autoclavable. Therefore, equipment with plastic and rubber parts that are not suitable for sterilization are more prone to biofilm development.

Studies performed by the BioProcess Technical Institute, University of Minnesota (summarized in Table 10.1), indicate that biofilms formed on Teflon, a type of material that can be easily cleaned, are easier to remove as compared to biofilms formed on other surfaces that may have imperfections and are harder to clean [18]. Indeed, cleanability properties of materials are important factors when considering whether a given material will be prone to biofouling or not.

Given the challenges in controlling microbial contamination in biopharmaceutical manufacturing equipment and materials, many companies have opted to use single-use or disposable items such as filter membranes, tubing/hoses, and connectors (e.g., Lynx ST, by Millipore Corporation, www.millipore.com). There is in fact a trend in biopharmaceutical manufacturing toward the use of disposable materials and even equipment, such as bioreactors. Although disposable systems are expensive, a fact that might be of concern to some companies, the initial cost is actually offset by not having expenses associated with sanitization (e.g., labor, power consumption, and water/chemicals), material life-cycle studies, cleaning validation, and biofilm remediation, the latter in case of contamination events.

BIOFILM CONTROL AND PREVENTION

Biofilm control involves one or more of the following strategies: preventing the initial contamination of the material, attempting to minimize the initial microbial adhesion to the surface, killing of the biofilm cells via chemical or heat treatment, and removing the piece of equipment altogether and replacing it with a new and clean one. Ideally, preventing the formation of biofilms would be a more logical approach than treating them.

Unfortunately, there is currently no available technology that can truly modify a surface to resist biofilm development without causing some adverse effect. In fact, attempts to prevent biofilm formation in industrial systems by manipulating the chemical composition of metallic materials and surface characteristics of pipes and vessels have failed. Therefore, the focus should be on optimizing equipment maintenance procedures to reduce the chance of microbial contamination as well as improving methods to kill and remove established biofilms.

Heat Treatment

Heat is very effective in removing biofilms, and it should be the first choice for biofilm prevention and remediation. Companies should modify, whenever possible, equipment components so the system can be steamed in place. In order to do so, the equipment must be fitted with steam traps, and should have parts and components that can withstand heat. Another alternative is to disassemble the various equipment parts, autoclave them, and then reassemble the equipment using aseptic technique and under aseptic conditions.

According to researchers at the Center for Biofilm Engineering (CBE; www.erc.montana.edu) at Montana State University in Bozeman, Montana, cold temperatures have no adverse impact on biofilms. Freezing has a major effect on ice crystal formation; however, water crystallization in the EPS matrix is quite muted. Therefore, although freezing of planktonic cells in water will kill them, freezing a biofilm will not destroy most of the sessile cells.

Chemical Treatment

Equipment that cannot undergo steaming in place or autoclaving must be chemically sanitized prior to use. As discussed in Chapter 6, chemical sanitization of equipment can be accomplished using caustic, acidic, and oxidizing agents such as hydrogen peroxide and sodium hypochlorite solutions. Oxidizing chemicals can actually dissolve the polysaccharide matrix and kill the bacteria. These solutions are very effective in biofilm removal but, unfortunately, not compatible with many materials.

The use of gluteraldehyde as a chemical sterilant for process equipment is not common in the pharmaceutical and biotech industries. Gluteraldehyde is also not very effective against biofilms because this chemical is actually used up in the matrix material, resulting in pickled biofilm. If the chemical sanitizer is not able to destroy all the cells in a biofilm before the chemical is removed, the surviving cells will be able to rapidly proliferate in the rich medium of cell debris resulting in the reestablishment of a full biofilm within a few hours.

Typical sanitizers used to clean and store biopharmaceutical equipment such as chromatography columns and UF/DF systems include sodium hydroxide (NaOH) solutions (0.1 M and 0.5 M). However, dilute NaOH solutions are not very effective in removing or killing established biofilms, and its use often results in pickled biofilm that is prone to rapid regrowth. Antimicrobial effectiveness studies performed by static exposure of less-resistant planktonic microorganisms to NaOH solutions confirmed the limited antimicrobial properties of dilute NaOH solutions. However, a 1.0 M NaOH

solution demonstrated greater antimicrobial effectiveness against vegetative cells but only limited reduction of bacterial spores after a prolonged contact time [19].

A study performed using biofilms of *P. aeruginosa* and *K. pneumoniae* identified some potential chemicals that can be used for biofilm removal and equipment cleaning. For the study, the biofilms grown in a biofilm reactor for 7–9 d were exposed to the test chemicals for a 1-h period. Treatments that caused removal of more than 25% of the biofilm mass (tested as total protein) included those by NaCl; $CaCl_2$; ethylenediamine-tetraacetic acid (EDTA) and Dequest 2006 (both chelating agents); the surfactants SDS, Tween 20, and Triton X-100; and an increase in pH, lysozyme, hypochlorite, monochloramine, and concentrated urea. Treatments that resulted in less than 25% removal of biofilm mass under the test conditions included those by $MgCl_2$, sucrose, nutrient upshifts and downshifts, and a pH decrease [14].

Although researchers have identified chemicals that are effective in killing and removing biofilms, when it comes to remediation of established biofilms formed in biopharmaceutical equipment, companies have limited choices; this is because many of the most effective chemicals are detrimental to equipment surface materials or to the type of process. Therefore, there is a need to develop alternative and more effective protocols for equipment cleaning and storage. For example, performing a chemical shock treatment (e.g., multiple short chemical sanitization cycles over a period of hours/days) instead of a single treatment lasting a few hours may be beneficial in completely eradicating a resident and well-established biofilm and may be less damaging to equipment surfaces. The addition of a surfactant to a cleaning solution can help dislodge biofilm cells, and the chemical removal of sessile/attached microbial cells can be enhanced by dynamic rinsing and recirculation of the sanitizer instead of static immersion [19].

The use of vaporized hydrogen peroxide (VPHP) for killing of biofilms has shown promising results. Recent studies performed by STERIS Corporation (Mentor, Ohio) demonstrated that VPHP is an effective biofilm decontaminant and its application is especially useful for heat sensitive materials and devices because in the vapor phase, hydrogen peroxide is less aggressive to surface materials as compared to liquid hydrogen peroxide [20].

Prevention of Biofilms

The primary rule for biofilm prevention is to store equipment and materials dry as cells cannot form biofilms in the absence of water or moisture. It is also critical for companies to use product contact equipment that are of sanitary design and can be easily cleaned. Care should be taken when connecting pipes, gauges, sensor probes, hoses, and other parts of equipment to ensure that connections do not create dead legs or dead zones where liquid can collect.

Product contact surfaces should be maintained smooth, with no imperfections or deterioration that would lead to microbial colonization. Corrosion of metals after exposure to water and chemicals is of great concern in the pharmaceutical and biopharmaceutical industries: water is the largest component used in pharmaceutical manufacturing and its use can lead to detrimental effects on metal surfaces such as rouging, corrosion, and biofilm formation. One study evaluated the effects of corrosive treatment on stainless steel surfaces and bacterial attachment. Samples of surface finishes (electropolished, steel-ball burnished, glass-beaded, acid-dipped,

steel-shot burnished, and sandblasted) were compared with mill finish controls to determine the variation in bacterial attachment on each finish. Exposure to the corrosive treatment conditions resulted in changes in the numbers of bacteria that attached to each type of surface finish. After exposure, significantly greater numbers of bacteria attached to steel-ball burnished and glass-beaded finishes, whereas the control mill finish and electropolished samples had fewer bacteria attached after exposure. The electropolished samples were significantly more resistant before and after exposure to corrosive treatment than the seven other finishes tested [21].

Much work has been carried out in the area of biofilm prevention as it relates to material surface properties. Some companies have applied the knowledge gained on physicochemical characteristics of microbial cells to create surfaces that claim resistance to microbial adhesion. Although most of this work has targeted medical device applications, some of the technologies could be applied to the pharmaceutical and biotech industries. For example, electrostatic repulsion has been used to create materials that have a zone of microbial repulsion; hydrogels applied to surfaces and polymer brushes (alginate, polyethylene glycol [PEG]) composed of a layer of polymers attached with one end to a surface have been used to prevent microbial adhesion. Studies with materials that alternate between hydrophobic and hydrophilic properties, with shifts in temperature, have also shown promising results [22]. Research on materials that are physical blends of active biocides and polymeric molecules as well as polymers or copolymers of a biocidal monomer has also been performed. However, more work is needed in this area because issues with biocide stability and with biocides leaching out of the materials still need to be resolved.

One area of research that seems to offer great possibilities for pharmaceutical applications is the use of immobilized agent systems such as cationic polymers (e.g., positively charged amines with associated hydrophobic regions). One such example is the ÆGIS Microbe Shield™ technology (www.theciagroup.com). During application, the material surface is modified by the formation of a permanent antimicrobial polymer—a clear and protective shield. This product carries a positive ionic charge. Because most microorganisms carry a negative charge, electrostatic attraction pulls the microorganism into direct contact with the active polymer. Once contact is established, the cells are destroyed by what could be compared to "a spear thrust followed by electrocution" (electromechanical kill mechanism).

Entegris, Inc. (www.entegris.com) developed a corrosion-resistant fluoropolymer material for use in biopharmaceutical processing called Fluoropure®. The fluoropolymer sheet can be used as a lining for vessels, tanks, bioreactors, fermentors, and chromatography columns of up to 80,000 L in capacity. This material has potential in enhancing cleanability and resistance to biofilm development. Other areas of research in biofilm prevention will be presented later in this chapter when the future of biofilm research is addressed.

METHODS FOR DETECTION AND RECOVERY OF BIOFILM ORGANISMS

As discussed earlier in this chapter, biofilm bacteria are phenotypically and metabolically different as compared to their planktonic counterparts. Therefore, tra-

ditional microbiological test methods are not suitable for detection or recovery of biofilm cells. The swabbing technique widely used in the pharmaceutical industry for sampling bioburden on surfaces may not adequately remove a portion of a biofilm for evaluation. Given the physical characteristics of a biofilm structure, with cells embedded in the EPS matrix, a microcolony that is picked up may become wrapped around the fibers of the swab and it might not be dislodged onto the test agar plate. If a portion of the biofilm does get dislodged from the swab and it contains perhaps hundreds of bacteria, it may grow as a single colony. Therefore, using the swab technique to quantify biofilm cells is not recommended.

The same problem occurs with rinse samples collected from equipment-cleaning verification studies; if the flow of the rinse liquid is slow, it will not be able to disrupt a well-established biofilm and, therefore, few to no cells will be detected. In case biofilm cells are recovered and plated using an all-purpose microbiological medium and standard incubation conditions for bacteria, the sessile cells may not grow to form colonies. The medium may be too rich or the incubation conditions may be unsuitable for biofilm cells to proliferate. In summary, traditional microbiological methods designed for testing planktonic and culturable microorganisms are not suitable for detection of most biofilm organisms.

There are basically two ways to analyze cells in a biofilm: removal or disaggregation followed by traditional microbial culturing methods and analysis of intact biofilms in situ. When attempting to remove biofilm cells, the best approach is to scrape the surface using a sterile and hard device such as a scalpel, wooden stick, or plastic loop. After the biofilm is removed, cells should be suspended in a sterile buffer followed by disaggregation of cells via sonication, vortexing, or homogenization, the latter producing the most consistent results. Once cells are suspended in the buffer medium, enumeration of biofilm organisms can be performed via standard methods such as plate count, agar overlay, membrane filtration, dry weight assay, and total protein count. In case a microbiological test is chosen, optimization of recovery methods must be carried out to ensure that appropriate medium and incubation conditions for the target organisms are used. Biofilm removal checks can be performed using dye tests such as crystal violet or safranin assay and the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) assay. A new method for distinguishing between live and dead bacteria was developed by Andreas Nocker, professor at the CBE. This method consists of a fast (approximately 10 min) and easy technique to treat a bacterial sample using the chemical propidium monoazide (PMA) that limits the diagnostic analysis to the live portion of mixed communities [23].

Intact biofilms can be detected on surfaces using techniques such as microscopy, dye binding, and autoradiography. A typical microscopic method used is light microscopy (dark field and phase contrast), which is good for examination of early stage biofilms. Fluorescence microscopy and confocal laser scanning microscopy also provide the ability to observe thick biofilms in situ.

Rapid methods can be useful for detection of biofilms in a timely manner. One example is the hydrogenase test for bacteria that cause metal corrosion. In order to corrode metals, these organisms must have the enzyme hydrogenase. Therefore, instead of enumerating the cells, the rapid method checks for enzyme levels evidenced by a color change that occurs in a minimum of 20 min (heavy biofilm) to

about 4 h (very thin biofilm). PCR (polymerase chain reaction) and bioluminescence techniques have also been used for biofilm detection and quantitation. Rapid methods, although still not widely used in the pharmaceutical microbiology laboratories mainly due to sample destruction during analysis, would be extremely valuable for the detection of biofilms in process equipment so that prompt corrective measures could be implemented, thus avoiding equipment downtime.

QUALIFICATION OF CHEMICAL SANITIZATION USING BIOFILM CELLS

Traditional test protocols for evaluating antimicrobial effectiveness of sanitizing and disinfectant solutions employ ideal laboratory conditions and cultures of free cells, (planktonic cells) thus allowing for excellent and uniform physical contact of the antimicrobial agent and the microbial cells that are metabolically active. The reality is that microbial cells in a biofilm are less active, and often the antimicrobial solution is unable to adequately penetrate the biofilm. Therefore, biofilms typically require 1000 times the concentration of an antimicrobial agent or a much longer contact time to be effective [2]. Given these facts, it should be clear that standard methods for testing sanitizers and disinfectants employing planktonic cells, which is described in the USP Chapter <1072> *Disinfectants* and *Antiseptics* and in the AOAC International [24] are not suitable for testing biocides against biofilms. Indeed, the metabolic state and phenotype of the test organisms used to measure the efficacy of antimicrobials in the laboratory are seldom equivalent to the problem-causing organisms found as surface contaminants.

Testing chemicals for their antimicrobial effectiveness against biofilms is performed using microbial cells grown in a biofilm reactor. Several model biofilm reactors have been developed at the CBE to simulate low- to high-shear environments. Although most biofilm methods are performed using single culture biofilms, evaluating the effects of sanitizing agents against mixed-culture biofilms is also recommended by the author; besides being a more prevalent type of microbial community in contaminated equipment, heterogeneous communities may exhibit different antimicrobial resistance patterns as compared to pure-culture biofilms.

High-throughput screening tests for sanitizers against biofilm cells have also been developed using microtiter plates. For example, a quantitative spectrophotometric assay was developed to measure the removal and killing efficacy of antimicrobial agents using 96-well plates [25]. Methods using microtiter plates yield high variability in test results, and are not suitable for distinguishing antimicrobial effectiveness of chemicals of close chemistries. Therefore, this type of method is not recommended for evaluation of antimicrobial properties of sanitizers prepared at different use concentrations.

It this chapter, the author will present various biofilm reactors that simulate high, low, and no shear environments and will discuss the current approaches to practical and reliable laboratory biofilm test methods for challenging sanitizers and disinfectants.

Types of Biofilm Reactors

There are various types of *flow-through biofilm reactors*, including the flow cell system, the drip flow biofilm reactor, the rotating disc reactor, the annular reactor, and

FIGURE 10.5 The Flow Cell System (mini-tube flow cell on microscope): a flat plate flow cell designed to accommodate coupons of various materials to study biofilms using microscopes. Other models are also available. (Photo courtesy of Paul Stoodley and Bryan Warwood, BioSurface Technologies Corporation, Bozeman. With permission.)

the CDC (Centers for Disease and Control) biofilm reactor. These devices, described in detail in the following text, were designed at the CBE (the CDC Biofilm Reactor was designed at the Centers for Disease Control and Prevention, Atlanta), and are manufactured by BioSurface Technologies Corporation (421 Griffin Drive #2, Bozeman, Montana; www.biofilms.biz). Other biofilm reactors with similar designs have been customized by companies for their specific applications.

The flow cell system (Figure 10.5): This system is designed to allow for visual observation (microscopy and image analysis) of biofilm development and growth in low- to medium-shear environments. All parts are reusable and autoclavable, and the optical viewing glass is compatible with upright and inverted light, epifluorescence, and confocal microscopes. One of the disadvantages of this system is its limited sampling capabilities—there is a very small fraction of test surface available for analysis, thus making this biofilm reactor not very practical.

The drip flow biofilm reactor (Figure 10.6): This is a robust system designed to evaluate biofilms formed under low-shear/slow laminar flow conditions. It consists of four parallel test channels, each capable of holding one standard microscope glass slide, or a length of a catheter or stint. The growth medium is provided by dripping (gravity) over the coupon or catheter surface. This unit is limited by the number of coupons and is more suitable for medical device applications.

The rotating disc reactor (Figure 10.7): This system is designed for laboratory evaluations of biocide efficacy and biofilm removal in high-shear/tur-

FIGURE 10.6 The drip flow biofilm reactor. (Photo courtesy of BioSurface Technologies Corporation, Bozeman. With permission.)

FIGURE 10.7 The rotating disc reactor. (Photo courtesy of BioSurface Technologies Corporation. With permission.)

FIGURE 10.8 The annular reactor. (Photo courtesy of BioSurface Technologies Corporation. With permission.)

bulent flow environments. It contains six removable coupons that can be made of various materials. All parts are reusable and autoclavable. A liquid growth medium (or biocide after biofilm is formed on the coupons) circulates through the vessel while mixing, and shear is generated by a magnetic stir bar rotated by a magnetic plate. This system is very reliable and has great repeatability. Some disadvantages of this biofilm reactor include the limited number of coupons and the fact that the entire coupon shaft has to be removed for evaluation, thus making it an endpoint test.

The annular reactor (Figure 10.8): This system is designed to evaluate biofouling and biocorrosion in water distribution systems under controlled shear conditions. A variable speed motor drives an inner rotating cylinder to provide surface shear to match the desired process conditions. There are 20 removable slide coupons that can be manufactured using any type of material; each coupon can be separately removed for biological evaluations, metal-loss corrosion studies, and biofilm evaluations. One model has a water jacket for precise temperature control. All parts are reusable and autoclavable. This system is very reliable and demonstrates great repeatability. One disadvantage is its high cost as compared to other biofilm reactors.

The CDC biofilm reactor (Figure 10.9): This system is designed to study biofilm development in high-shear/turbulent environments. The reactor is completely autoclavable and reusable. It has capability to test a total of 24 coupons, made of various materials, using time-course studies. A liquid growth medium (or biocide after the biofilm is formed on the coupons) is circulated through the vessel while mixing, and shear is generated by a

FIGURE 10.9 The CDC biofilm reactor. (Photo courtesy of BioSurface Technologies Corporation. With permission.)

magnetic stir bar rotated by a magnetic plate. This unit is licensed from the CDC, and it has been shown to be very robust. The CDC biofilm reactor can be very reproducible when optimized for the organism under evaluation.

The static biofilm reactor (Figure 10.10): A method for creating biofilms for evaluating efficacy of disinfectants under no shear/no flow conditions was developed by Charaf based on the methods approved for disinfectant efficacy testing as described in the AOAC [26]. This method, which is often referred to as the *static biofilm reactor* or the *colony biofilm model*, utilizes basic laboratory supplies. Testing using the Static Biofilm Reactor has been proven simple and reproducible.

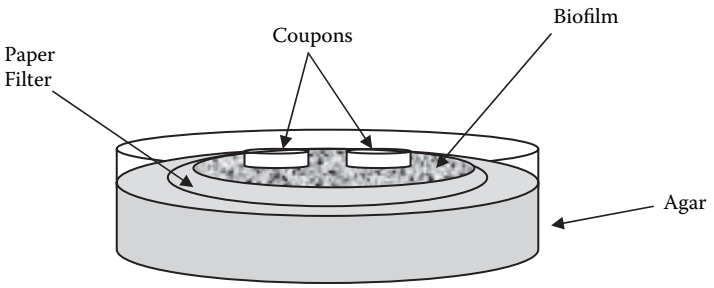

FIGURE 10.10 The Static Biofilm Reactor.

Studies performed to compare biofilm recovery methods and biofilm resistance to disinfectants using the drip flow, CDC, and static biofilm reactors demonstrated that fluid dynamics not only influence biofilm formation but also its resistance to biocides. Biofilms developed under no shear conditions were not strong, were easily removed through washings, and were much more susceptible to disinfectants. The strongest biofilms were created using the CDC biofilm reactor, and the cells were hardier or much more resistance to the biocides and more difficult to be removed [27, 28].

Although much work has been done in the area of standardization of biofilm populations in a laboratory setting and biocide testing against biofilm cells, to this date, only two biofilm reactors and standard operating procedures have been approved by the American Society for Testing and Materials (ASTM) International—ASTM E-2196-02 [29] and ASTM E-2567-07 [30]. The E-2196-02 method employs the rotating disc biofilm reactor, which was created as a model to study biofilms in toilet bowls. The E-2567-07 method employs the CDC biofilm reactor to grow, sample, and analyze *P. aeruginosa* biofilms grown under high-shear conditions. Indeed, the practice of testing disinfectant against biofilms has just begun. Therefore, it behooves companies to be proactive in anticipation of further standardized biofilms methods that may eventually be referenced in the compendia and/or regulatory guidance documents.

Choosing a Biofilm Reactor

When choosing a biofilm reactor and the test microbial growth conditions, one must select the system that will represent the best model for the equipment and process environment of interest. Indeed, it is a challenge to design a laboratory system for evaluation of biocide efficacy against biofilm cells—one must balance the relevance of the system and its practicality while ensuring accuracy and reproducibility of the data generated because inherent variability in microbiological testing can directly impact test results. Therefore, studies must include optimization of the biofilm reactors and of the chosen microbial recovery methods and sufficient test replicates to determine parameters such as ruggedness, sensitivity, repeatability, reproducibility, bias, and variability. Based on information gathered and evaluations performed by the author, the CDC biofilm reactor appears to be the most appropriate for evaluation of antimicrobial efficacy of solutions used for biopharmaceutical equipment cleaning and storage. A practical approach to growing biofilms and testing disinfectants using this type of biofilm reactor is described in the following section.

Testing Sanitizers Using the CDC Biofilm Reactor

Testing disinfectants and sanitizers against biofilm bacteria begins by growing representative microbial contaminants or environmental isolates in a well-controlled biofilm reactor that contains coupons made of various types of equipment and material surfaces (e.g., Teflon®, glass, PP, acrylic, etc.). See Figure 10.11 for a schematic of a typical CDC biofilm reactor setup when continuous flow studies are performed.

Setting up the Biofilm Reactor

A typical protocol for growing single-culture biofilms involves inoculation of the bioreactor containing the sterile growth medium (typically 350 mL of tryptic soy broth

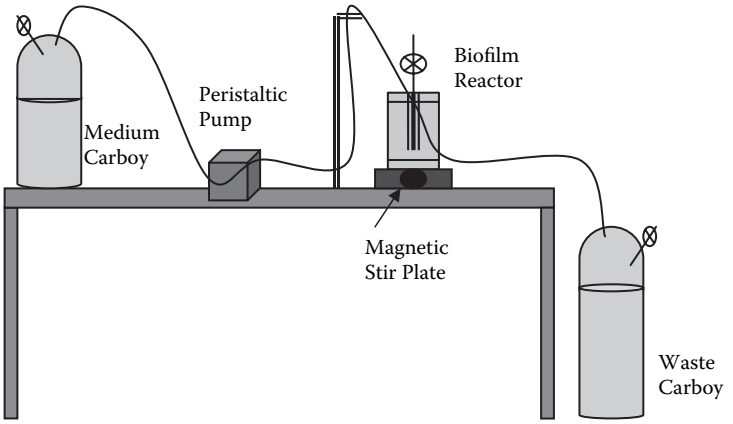

FIGURE 10.11 Schematic of a typical setup for the CDC biofilm reactor.

[TSB]) and the test coupons with 1mL of an overnight culture of the test organism (Figure 10.12, step 1). A standard protocol calls for the use of six coupons of each type of material: three will be exposed to the biocide dilution and three will be used as positive controls. Prior to use, coupons should be inspected visually for scratches and other flaws. Defective coupons should be discarded.

The biofilm reactor is first set up to operate in batch mode for about 24 h at the desired test temperature that would mimic the environment in which the biofilm would be formed. During this incubation period, the medium is constantly stirred to create the desired shear. Rotation of the shaft containing the test coupons is usually set between 125–200 rpm for studies designed to generate medium to high shear. At the end of this initial incubation period, the microbial cells would have attached to the surface of coupons and the initial phases of biofilm formation would have been initiated.

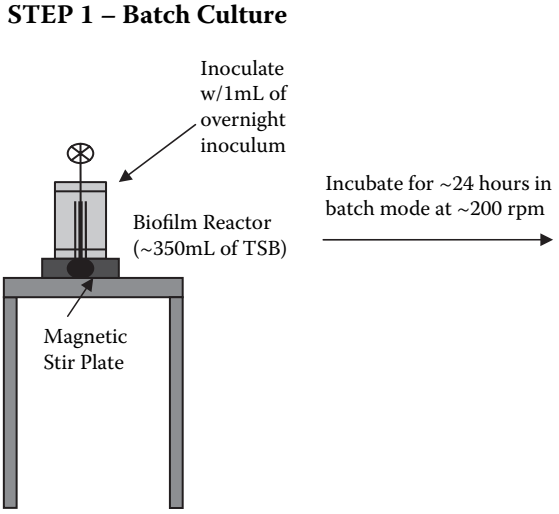

FIGURE 10.12 Testing sanitizers using the CDC Biofilm Reactor. (Continued)

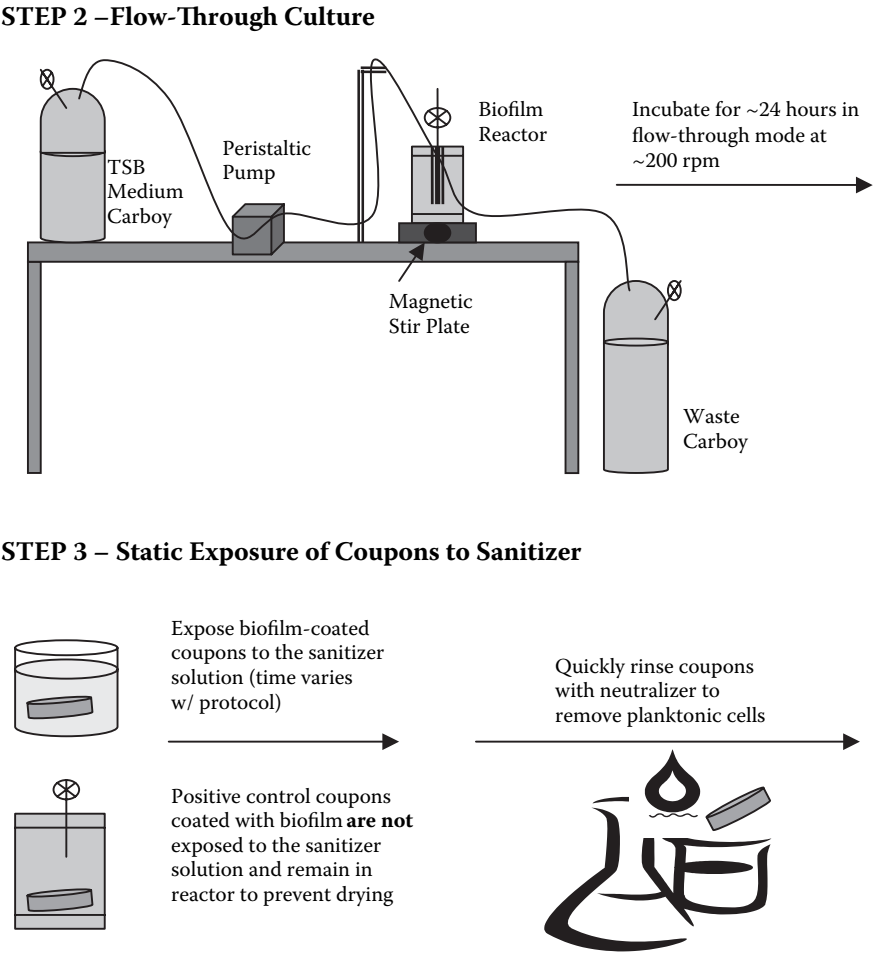

FIGURE 10.12 Continued.

Following the initial 24-h batch mode incubation period, the bioreactor is set up for a continuous flow operation, at the desired flow rate, for another 24 h and at the same selected temperature (Figure 10.12, step 2). During this phase of the procedure, the growth medium is continuously replaced with fresh medium while being stirred to create the desired shear. In order to ensure biofilm formation and establishment of sessile cells, the dilution rate of the growth medium must be controlled so that the doubling time of the microorganisms is much higher than the residence time (time it takes for the bulk fluid to be replaced in the reactor). This will ensure that planktonic cells are washed out whereas biofilm cells remain attached to the coupons.

The residence time can be calculated as follows:

$$\text{Residence time (RT)} = \frac{\text{Volume of medium in reactor (mL)}}{\text{Flow rate (mL/min)}}$$

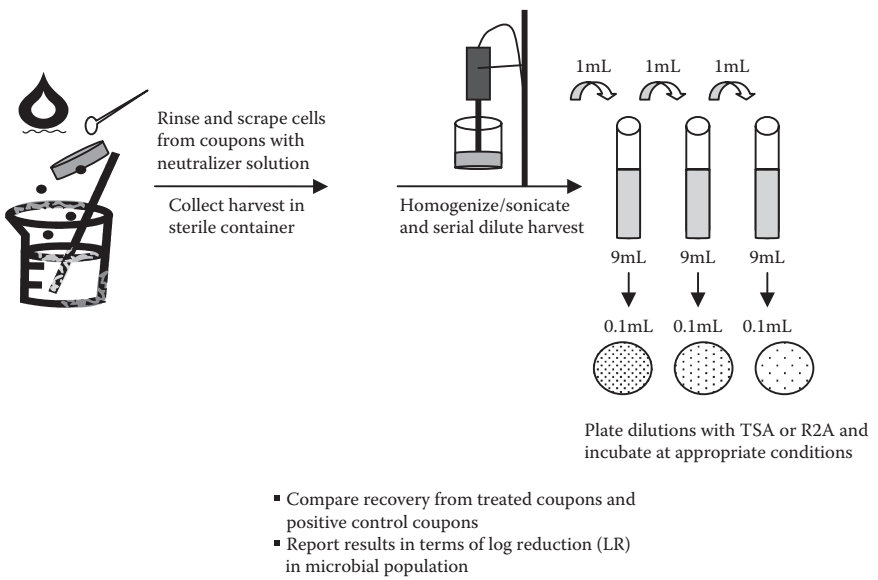

FIGURE 10.12 Continued.

Exposure of Biofilm to Disinfectant Solution

At the end of the 48-h incubation period, the biofilm formed on the surface of the coupons is ready for harvest and exposure to the test sanitizer/disinfectant (Figure 10.12, steps 3 and 4). The biofilm cells from the three coupons used as positive controls are harvested without being exposed to the sanitizer. The other three test coupons are exposed to the sanitizer solution for a defined period of time (typically 5 to 10 min) prior to harvest. Exposure can be *static*: immersing the biofilm coated coupons in a container with the sanitizer; or under *dynamic flow conditions*: leaving the biofilm-coated coupons in the biofilm reactor, set up the reactor for flow-through mode, and replace the enrichment medium with the chosen sanitizer/disinfectant solution.

Harvesting Biofilm Cells

Prior to scraping and rinsing the biofilm cells off the coupons (exposed to biocide and positive controls), briefly rinse the coupons with a sterile neutralizer buffer solution to remove planktonic and loosely attached cells. Then, biofilm cells are harvested from each coupon separately, via scraping and rinsing the microbial growth with the same sterile neutralizer buffer solution (total rinse volume is about 10 mL). Use a sterile scalpel or wood stick to scrape the biofilm cells from the test coupons. The neutralizing rinse and cells are collected into a sterile container and the microbial suspension is homogenized or sonicated for approximately 30 seconds to disaggregate the biofilm cells. The sample preparations are then serial diluted for plating and enumeration of viable cells using optimized microbiological test methods. Typical recovery media used are TSA and R2A with incubation at 30–35°C for 24–48

h. After incubation, the recovered colonies are enumerated and the density of viable bacteria on each coupon is calculated. When performing the calculations, use the test dilution that yields counts between 25–250 CFU. If no counts are recovered for the lowest dilution, results are reported either as 0 CFU or < 1 CFU per coupon.

Sanitizer/Disinfectant Efficacy Evaluation

Following calculation of recovered number of viable cells, the microbial density reported for each coupon is $\log_{10}$-transformed. The disinfectant/sanitizer efficacy is determined by comparing the average number of viable cells remaining on the biocide-treated coupons to the average number of viable cells on the untreated coupons (positive control). Antimicrobial efficacy is determined as log reduction (LR) in microbial population: the mean log density calculated for the positive control minus the mean log density calculated for the corresponding biocide-treated coupons.

Method Qualification and Test Controls

The procedure used for growing biofilms in the laboratory must be validated to ensure accuracy and reproducibility of test results. Biofilms formed on the test coupons (see Figure 10.13) are subjected to various types of manipulations during harvest, disaggregation, biocide exposure, and neutralization after exposure to a biocide. All these test manipulations must be standardized in order to achieve reproducible results. There is always the potential for cell wash-off during the rinse/neutralization step, and increased plate count variability if cell harvesting is not optimized. In general, a method is found suitable when biofilm density recovered from the test-positive

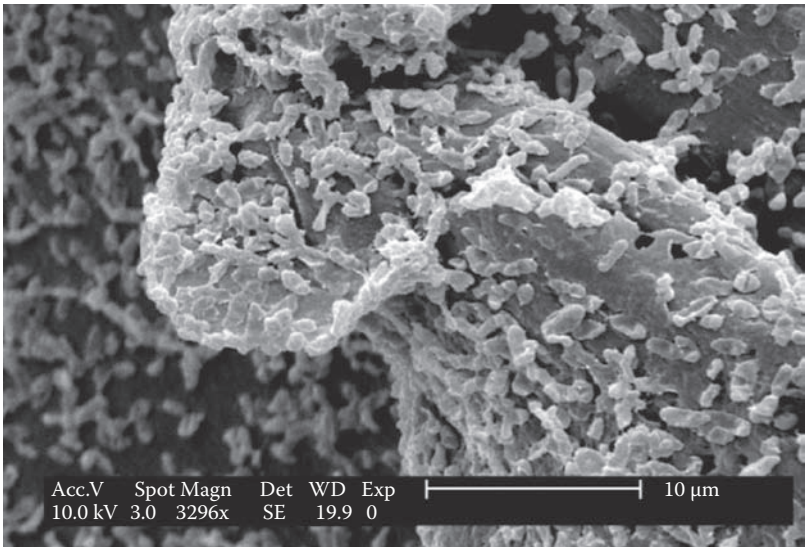

FIGURE 10.13 Scanning electron micrograph of a *P. mirabilis* (ATCC 29906) biofilm growing on PC (polycarbonate) coupons using a CDC biofilm reactor. (From Public Health Image Library, Center for Disease Control and Prevention, U.S. Department of Health and Human Services. With permission.)

TABLE 10.2
Effect of Various Biocides on Planktonic and Biofilm Organisms Using the AOAC Qualitative Germicidal Spray Test

Chemical Sanitizer	*P. aeruginosa* (Planktonic) # Positive/Total Coupons	*P. aeruginosa* (Biofilm) # Positive/Total Coupons	*S. aureus* (Planktonic) # Positive/Total Coupons	*S. aureus* (Biofilm) # Positive/Total Coupons
Sodium hypochlorite (1000 ppm)	0/60	60/60	0/60	39/60[a]
Sodium hypochlorite (1000 ppm) (Formula #1)	0/60	59/60	0/60	60/60
Sodium hypochlorite (1000 ppm) (Formula #2)	0/60	60/60	0/60	60/60

Note: Passing results: ≤1 positive/60 coupons.

[a] Samples may not have been neutralized properly.

Source: Adapted from www.biofilmsonline.com/cgi-bin/biofilmsonline/ed_static_glass_plateA.

controls is within 10^7–10^8 CFU/coupon and reproducibility among the three test replicates is within 0.5 log of the calculated mean value.

Testing Sanitizers Using a Static Biofilm Reactor

To set up a static biofilm reactor (Figure 10.10), an overnight culture of the test organism is inoculated onto a sterile filter paper that is placed on top of an agar plate (e.g., TSA). Then, sterile flat coupons are placed onto the inoculated filter paper and the sample preparation is incubated at suitable conditions that best represent the environment where the biofilm would be formed. As the biofilm grows within the filter, it also covers the underside of the test coupons. Typically, biofilms are ready for harvest after a 48-h incubation period. Exposure of coupons to a test disinfectant and recovery of the biofilm follow the AOAC International Test Method 960.06 [24]. The Static Biofilm Reactor is a good biofilm model for membrane-like surfaces (porous surfaces) such as wound dressings.

Studies performed using the traditional AOAC qualitative Germicidal Spray Test using planktonic cells and the modified AOAC method using biofilms created by the Static Biofilm Reactor confirm that biofilm organisms are much more resistant to antimicrobial products [26]. A summary of the test results obtained is presented in Table 10.2. The differences in kill between planktonic and biofilm cells (less hardy/formed under no shear conditions) are quite significant and clearly demonstrate the need for companies to test their equipment sanitization and disinfectant solutions using appropriate models of biofilm reactors.

INDUSTRIAL SIGNIFICANCE OF BIOFILMS

The ability to reliably control bioburden levels in a process stream is critical in pharmaceutical manufacturing, especially, in biotechnology processing where many of

the downstream unit operations are carried out in a bioburden controlled manner. Microbial contamination in an upstream process step may raise quality concerns but may be tolerated because further downstream processing often reduces bioburden to acceptable levels. However, in a downstream or final process step, a microbial contamination event may lead to rejection of a product batch.

Microbial biofilms cost companies thousands to millions of dollars annually in equipment damage, production downtime, product contamination, investigations, remediation, and energy losses. As conventional methods for killing planktonic bacteria are often ineffective when applied to biofilms, once biofilms are established, routine equipment-cleaning and sanitization procedures become ineffective and contamination events may linger for months if not years.

Biofilms that form in equipment and on materials used in the production of pharmaceutical products pose a safety risk even when product bioburden levels meet specifications. The presence of a biofilm of Gram-negative bacteria in equipment or materials used in the manufacturing of parenteral products can potentially lead to high levels of endotoxin, resulting in rejection of product batches or costly remediation of WFI systems. There are also concerns with other toxic products that may be produced by biofilm cells but not screened for nor eliminated during the various process unit operations. Indeed, this is a great concern in pharmaceutical manufacturing because a biofilm may be established in a piece of equipment with direct product contact, shedding toxic products, and traditional bioburden grab samples may not detect the presence of the sessile cells.

As one can see, it behooves a company to be diligent about contamination control and to gain a better understanding of how biofilms are established and how they thrive. Indeed, it is better to take a proactive approach in the prevention of microbial contamination rather than trying to eradicate the biofilms once formed.

THE FUTURE IN BIOFILM RESEARCH

An area of concern for pharmaceutical manufacturers is that even with validated cleaning and sanitization procedures consistent with good manufacturing practices, microorganisms can remain on equipment and other production surfaces, and eventually grow into biofilms. This presents a challenge to companies, especially in controlling potential microbial pathogens. Much effort has been focused on developing alternative strategies (in addition to routine cleaning and sanitization) to further minimize the potential for biofilm development and to improve the test methods for verification of sanitizer efficacy.

Research on biofilms continues on many fronts with particular interest on genes that are specifically expressed by biofilm-associated microorganisms. Besides gene regulation, hot topics in biofilm research include EPS structure, quorum sensing, cyclic-di-GMP, enzymes, and phenotypic variants and persister cells. Research in the field of enzymology is targeting enzymes that can destroy the EPS matrix as a means of biofilm removal Studies performed using polysaccharide-degrading enzymes have indicated that pectin esterase is very effective in the removal of biofilm cells. Other enzymes, to include pronase and pectin lyase, have also shown promising biofilm removal capabilities [31]. Studies performed at the CBE with cyclic-di-

GMP, a chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels, have promising applications. Test results indicate that cyclic-di-GMP inhibits *S. aureus* cell–cell interactions and biofilm formation. Additional studies in this area of research are continuing to further evaluate the spectrum of biofilm inhibition by this cyclic di-nucleotide compound.

Several other recent studies have been performed on a molecular level to better understand biofilm development. In an article published in the *Journal of Bacteriology* in 2004 [32], studies to evaluate biofilm formation and sporulation were performed using *Bacillus subtilis* not only as single cells but also as cell communities. The data generated provided valuable information regarding the genetic control of biofilm formation by *B. subtilis* in diverse settings along with some surprising findings. For example, spore formation, which was long thought to be a process involving only single cells, was proved to be closely associated with the development of multicellular communities. Another important finding was that some of the signals that regulate sporulation also regulate biofilm formation: the transcription factors Spo0A and sigma-H, both key regulators of the initial steps of sporulation, were shown to play a critical role in biofilm development, although sporulation itself is not required for establishment of a biofilm. The study performed using *B. subtilis* also demonstrated that the diversity of phenotypes observed for cultures grown in liquid and solid media resulted from the fact that this organism is able to utilize different pathways to form biofilms depending on the environmental conditions present.

Gram-negative bacteria also respond to nutrient limitation and other environmental stresses by synthesizing sigma factors. For example, in *Escherichia coli*, sigma factors are under the control of the *rpoS* regulon that regulates the transcription of genes to overcome the effects of stress. Because *rpoS* is activated during the phase characterized by slow microbial growth, researchers believe that the conditions that contribute to slow growth may also favor biofilm formation.

Given the fact that the first step in biofilm formation is actually the ability to attach to a surface and not microcolony formation or production of an EPS matrix, it is critical for future research projects to investigate the biological pathways used by bacteria to detect the presence of surfaces. Although the initial contact with a surface is not necessarily regulated and may happen by chance, there is evidence that formation of a stable cell–surface interaction may be regulated, that is, there exist genes that promote stable cell–surface interactions. One example is the Cpx signaling system of *E. coli*, composed of CpxA (a sensor kinase and phosphatase) and CpxR (a response regulator) that plays a role in surface detection and is known to regulate P-pili (which may play a role in surface adhesion) [33]. Therefore, it is worth evaluating whether similar surface-sensing pathways are required for biofilm formation by other species of bacteria. If one is able to control surface detection and the ability to initiate surface colonization, all the other pathways to complete biofilm formation would be negated.

Another area of research that seems to be promising involves *biosignal blockers* that are used to manipulate the behavior of bacteria, rather than killing them. Scientists have discovered many examples in nature where bacterial biofilm is suppressed by organisms that use biosignal blockers. One example is the Australian red algae *Delisea pulchra*, which never develops biofilms on its leaves. About a decade ago,

scientists discovered that this seaweed produces a class of chemicals called *furanones* that have a structure similar to the chemical signals used by bacteria to trigger quorum sensing. So in the presence of furanones, bacteria become "deafened," and cannot detect the chemical signals from other bacteria. To date, over 40 furanone compounds have been isolated, and some companies are working at developing industrial and medical applications for this discovery. For example, research and development for furanone-based marine antifouling paints, furanone-incorporated industrial products, as well as the use of furanones for treatment of illnesses such as cystic fibrosis are currently being done. There are some concerns over the use of furanones, and those are under investigation. One concern deals with the fact that furanones may have long-term adverse effects in humans if used for treatment of long-term illnesses. Confirmation of safety profile for many furanone compounds is therefore needed if such products are to be used in the healthcare industry. Another concern is the fact that furanones are not effective in disrupting an established biofilm. However, it appears that this limitation may have already been resolved by the discovery of a separate chemical signaling system that triggers biofilm detachment [7].

It is certain that new features about biofilms will be discovered as research on this subject continues. For now, pharmaceutical and biotech companies must use the knowledge gained thus far on the biology of biofilms to develop better microbial detection methods for sessile cells, better methods to challenge biocides, and improved biofilm remediation procedures. The use of process risk assessment tools such as Failure Mode and Effect Analysis (FMEA) and Hazard Analysis Critical Control Point (HACCP) is highly recommended in order to identify areas in the process and types of equipment that are under the high risk of becoming contaminated and developing biofilms. Through education of employees and problem solving/risk analysis techniques, pharmaceutical companies can create innovative approaches and develop effective procedures for biofilm prevention and removal. Until more knowledge is gained in this area, management of biofilms in the pharmaceutical and biotechnology industries may involve trial and error and continuous improvement approaches.

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11 Handling Aberrant and Out-of-Specification Microbial Data

Microbiological testing is performed in all phases of a drug product manufacture—from testing of raw materials and sampling of the environment where production takes place to testing of in-process, final drug product, and stability samples. Microbial testing also supports process and cleaning validation activities. All testing performed in support of both finished pharmaceutical products and active pharmaceutical ingredients (APIs) must comply with cGMP regulations as specified in the 21 CFR Parts 210 and 211, Subparts I (Laboratory Controls) and J (Records and Reports), and in FDA's document Q7, Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients (ICH Q7). These regulatory documents state that any out-of-specification (OOS) result obtained must be adequately investigated whether the material is acceptable for release or not. OOS data is defined as results that fall outside established product specifications or test acceptance criteria.

In this chapter, the author addresses laboratory investigations of *OOS results* and aberrant data; the latter is defined as any unexplained discrepancy in test result or significant deviation from expected result. The focus will be on investigations into questionable results obtained from microbiological analysis.

HISTORICAL OVERVIEW OF INVESTIGATING OOS RESULTS

The cGMPs for the pharmaceutical industry started back in 1962. Since then, regulatory expectation has been that laboratory testing be carried out using scientifically sound methods and that companies ensure the validity of the test results generated. However, it was not until the court case *United States vs. Barr Laboratories* in 1993 that new standards and protocols for the proper handling of aberrant and failing data generated in a pharmaceutical laboratory were established. The Barr case, as it came to be known, was discussed and studied by many people in the pharmaceutical industry not only during internal company meetings but also during national and international trade conferences. This court case also initiated the groundwork for a new way of addressing OOS and unexpected test results. Judge Wolin, who presided

in the case, provided specific guidance on how to handle aberrant data, laboratory errors, and OOS results, and he emphasized the importance of formal laboratory investigations in order to identify the source of the OOS data. For the first time, a timeframe for completing laboratory investigations was provided as guidance to the industry: the court stated that all investigations should be completed within 30 business days of the problem's occurrence.

In response to the court's ruling and industry needs, the FDA in 1998 issued a draft guidance document for industry for investigating OOS test results. This document, entitled *Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production,* and finalized in 2006, represents the FDA's current thinking on the topic of OOS investigations [1]. This document also attempts to clarify regulatory expectations on the topic of OOS data to achieve greater consistency in laboratory investigations throughout the industry.

Out-of-Specification (OOS) Result

The term *out-of-specification* was coined by Judge Wolin, who preferred to refer to a failing result as *out-of-specification* rather than *product failure*, the latter more commonly used by FDA investigators. He stated that companies must first thoroughly investigate an aberrant result before making a decision as to whether the result truly represents a product failure. A company can prove that an OOS result is not a product failure if it can be explained through laboratory investigation as being a laboratory error, if it can be excluded by a statistical outlier test, or if it can be overcome by retesting. This was indeed a turning point for management of QC laboratory data and one that created many repercussions throughout the pharmaceutical industry. Nowadays, practically every pharmaceutical company has a standard operating procedure (SOP) for investigating anomalous and failing test results.

An OOS result is generated when a material/product undergoing in-process, release, or stability testing fails to meet an expected result, specification, or acceptance criterion provided in batch records, test methods, or other company-approved documents.

OOS results fall into three main categories:

- Laboratory errors: Mistakes made during analysis of the product. These can be associated with calculation errors and/or failure to comply with test methods.
- Nonprocess-related or operator errors: These are errors made by operators during a manufacturing process but that may not affect the quality of the product.
- Process-related or manufacturing errors: These are errors associated with mishandling or mismanagement of the production process and include use of wrong equipment or errors in the manufacturing parameters. These types of mistakes can directly impact the quality of the product manufactured.

When an OOS is generated, regulatory expectation is for the firm to conduct a thorough laboratory investigation to determine whether the result is valid or not before the OOS result is invalidated and the test repeated. Contrary to the FDA guidance

document on OOS investigations, the Q7 *Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients* [2] states that investigations *are not normally needed* for in-process tests that are performed for monitoring and/or adjusting the manufacturing process. Many in the industry agree that this statement should really only apply during process validation studies where interim limits are in place. However, even under these circumstances, it behooves a company to perform a formal laboratory investigation to evaluate the accuracy of the test result in question in order to set up appropriate corrective measures.

In terms of microbiological testing, it is not uncommon for anomalous or aberrant results to be generated. Microbial testing is prone to greater-than-normal testing variability, and often a result must be evaluated and interpreted rather than taken at its face value. The difficulties and challenges associated with interpretation of microbial data are discussed in the USP Chapter <1117>, *Microbiological Best Laboratory Practices,* where it is stated that troubleshooting microbial excursion requires special training and knowledge in microbiology. Indeed, the QC microbiology laboratory sets itself apart from other QC operations, and therefore, unique and specialized technical training is expected for laboratory management, especially in the area of data review and interpretation.

To illustrate the difficulties associated with microbial testing, the author provides a list of potential aberrant results that could be generated when performing a microbial limit or bioburden test:

- Questionable or inconsistent recovery of organisms (e.g., duplicate plate count recoveries exceeding 25% of the mean)
- Inoculated sample plate counts that yield more than 0.5 log variability (0.3 log harmonized) from the actual inoculum used
- Microbial growth observed for test-negative controls
- Lack of microbial growth for positive control plates
- Media that do not meet growth promotion requirements
- Microbial growth observed on selective agars streaked with aliquots from enrichment broths that are clear (no visual microbial growth).
- Microbial growth observed in enrichment broths but no growth obtained on subcultures using nonselective media (e.g., TSA)
- Microbial identification results that cannot be explained (e.g., isolation of a bacterium from an artic ocean in a process sample)
- Microbial growth found only on one of the replicate test sample plates

LABORATORY INVESTIGATIONS

The FDA expects companies to perform laboratory investigations not only in case of OOS results but also when an out-of-trend (OOT) result is obtained so that the company can identify potential changes to a process or procedure. Investigations into aberrant/OOS results must be driven by a company-approved procedure that includes defined responsibilities for the analyst who generated the questionable result and the supervisor. This procedure should also describe the steps to be followed for a

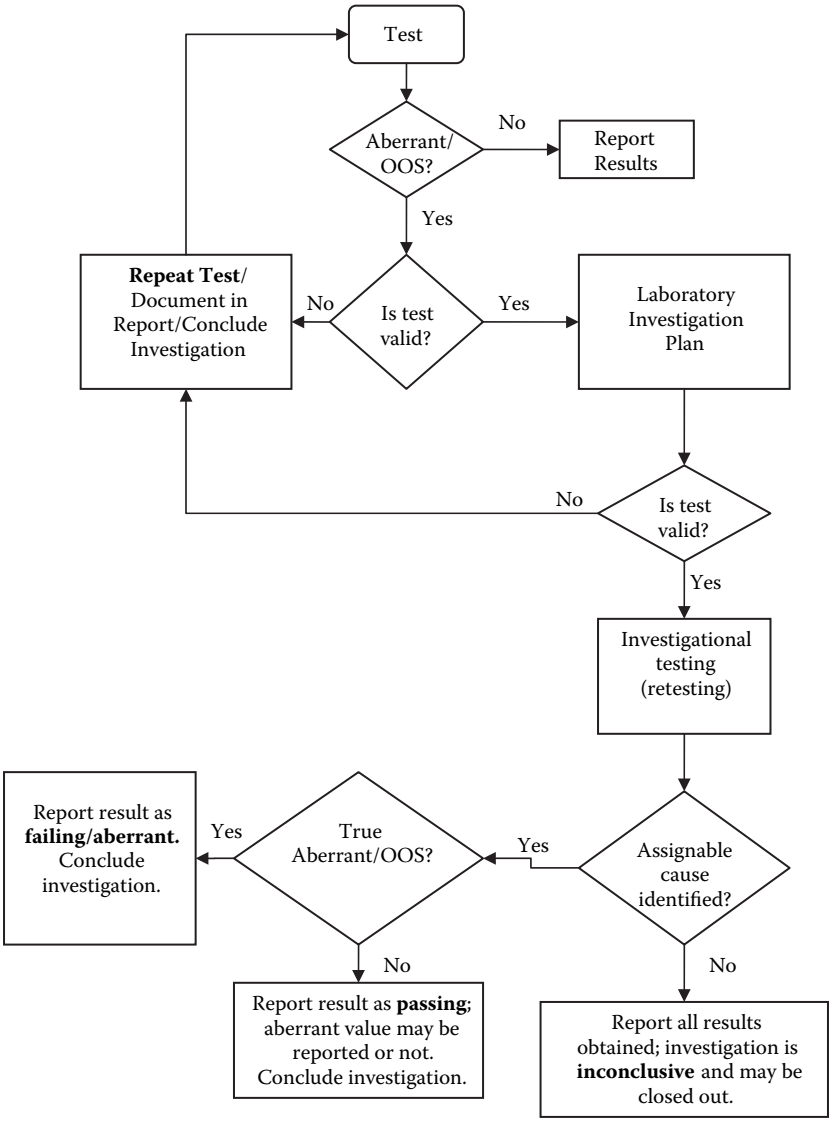

FIGURE 11.1 Flowchart for investigating OOS and aberrant results.

proper investigation to be conducted and for the proper documentation of the events. A flow diagram as shown in Figure 11.1 can be useful to understanding the flow of activities to be performed. The use of a checklist can also be beneficial when conducting a laboratory investigation. Figure 11.2 is an example of a checklist that can be incorporated in a laboratory investigation procedure for evaluation of OOS data from a microbial limit or bioburden test.

Because microbiological testing is quite different from chemical or physical analysis in terms of assay variability and potential for anomalous results, most general laboratory investigation procedures do not apply to microbial testing. For

- ☐ Visual examination of sample and container (e.g., discoloration, integrity breached, etc.)
- ☐ Compliance with test procedure to include use of appropriate test specification
- ☐ Analyst training status
- ☐ Check for calculation errors
- ☐ Quality control of media, buffers, diluents, and sterile materials used
- ☐ Equipment calibration and maintenance status
- ☐ Verification of incubation temperature and conditions
- ☐ Verification of results for test controls (positive and negative controls)
- ☐ Hood/aseptic manipulations monitoring (hood air monitoring/personnel) results
- ☐ Evaluate product testing history (stability data, if available)
- ☐ Evaluate test results for other steps in the process (in-process samples)
- ☐ Evaluate test results for other media used (if applicable)
- ☐ Identification of isolated organism (possible source of contamination)
- ☐ Evaluation of retesting/investigation testing results (if applicable)
- ☐ Calculation for outlier value (if applicable)
- ☐ Overall evaluation of data generated: does it make sense?

FIGURE 11.2 Investigation checklist for microbial limits and bioburden tests.

example, many laboratory investigations involve testing of sample retains to confirm a questionable result. However, when dealing with excursions from microbial tests performed for in-process and other perishable samples, the validity of results from retain samples is often put into question because test results are not typically available until 2 d after testing, and microorganisms can lose viability, or may proliferate during storage. However, microbial testing of retain samples could be performed on a case-by-case basis to provide limited information in support of the laboratory investigation. Other examples include the use of averaging of QC data and statistical calculations for outlier values. According to the FDA, there are both appropriate and inappropriate uses of the practice to average results from original as well as retesting data. For many chemical assays, such as testing for content uniformity, averaging of results is viewed as an inappropriate practice, and it may be discouraged in a general laboratory investigation SOP. However, in case of microbiological analysis, the use of averages is actually preferred by the USP and viewed by the FDA as an appropriate practice based on the inherent variability associated with microbial testing. Outlier testing is performed using statistical calculations to identify from a set of data results that are extreme. As in the case of averaging of data points, testing for outliers from chemical assays, is not a common practice. However, for microbiological assays,

the use of an outlier test is viewed as an appropriate routine practice during data analysis, even when dealing with data generated from validated/qualified methods. In the USP Chapter <111>, *Design and Analysis of Biological Assays*, outlier tests applicable to biological assays are described. The USP recommends that in case an outlier is confirmed, it should be omitted from sample calculations. Based on all the facts presented as well as personal practical experience, the author recommends that companies create a unique procedure for investigating OOS, OOT, and anomalous microbiological data. This document can be used in conjunction with the company general procedure dealing with OOS data, or as a stand-alone document.

Good documentation practices enhance the quality of an investigation, besides being a regulatory requirement for work performed in a cGMP environment; use of scrap paper, loose notebook paper, oral instructions, and other nontraceable information are not acceptable. Saving sample preparations/dilutions is also critical, especially during microbial analysis. Petri dishes, broths, and isolates must be preserved (preferably stored under refrigerated conditions) in case they are needed as evidence during an investigation and discarded only when the data have been reviewed and approved. A thorough and well-documented investigation will certainly pass regulatory scrutiny during an inspection. We all know too well that to regulatory inspectors and auditors, "if it is not in writing, it is a rumor."

Conducting the Investigation

The first step during a laboratory investigation is to verify and report the aberrant/OOS result to laboratory management; the analyst should have the questionable result promptly verified by a coworker or supervisor. If management is not available at the time the anomalous result is generated, an interview of the technician by his/her supervisor should take place within 24 h from the time the deviation is first observed. During this initial communication and evaluation of events, the supervisor gathers general information and confirms observance of the OOS result by examining evidence, compliance with test procedure, and verification of calculations (if applicable).

Management's assessment should be objective and timely, and should not contain any preconceived assumptions. Once the initial interview is complete, all evidence for any probable cause must be protected and promptly documented by the technician involved with the deviation or the laboratory supervisor/lead investigator. If during this initial phase of investigation there is conclusive evidence that the test performed is not valid due to an obvious laboratory error, the original result may be invalidated, and the test repeated. Typically, in such cases an invalid assay report is filled out. Alternatively, if the company procedure requires initiation of a laboratory investigation report at the time the anomalous/OOS result was first observed, the investigation can be closed out with recommendation for a repeat test to be performed. If during the initial data review there is no conclusive evidence as to the reason for the aberrant/OOS result, a *laboratory investigation plan* should be created, which should be approved by microbiology management and quality assurance prior to its execution. The laboratory investigation plan should minimally include the following:

- Reason for the investigation
- Summary of the procedure performed and sequence of events that may have led to the anomalous/OOS result
- Examination of equipment and instruments used during the test to check for calibration and preventive maintenance status
- A thorough review of the raw data to check for discrepancies
- Evaluation of the technician's training status
- Historical review of similar test results
- Review of other related tests performed for the product sample in question
- Review of stability data for product lot in question (if applicable)
- Proposed retesting procedure, to include sample aliquots and number of samples to be tested (investigational testing)
- Summary of test results and investigational testing
- A final quality assurance review, with approval signatures of all personnel involved with the investigation.

The investigation performed must be timely, thorough, and well documented. Laboratory management should give activities associated with the investigation the highest priority. If during the course of the investigation an assignable cause is found that explains the aberrant/OOS result as laboratory error, the investigation can be closed out; the original result can then be deemed invalid, and the test repeated. Examples of laboratory error that may invalidate the original test result include media/materials contamination, instrument malfunction, equipment used was out of calibration, sample was collected improperly by manufacturing, etc. If at the completion of the investigation plan the reason for the aberrant/OOS result is unknown, additional investigational testing may be warranted to assess whether the original result could possibly be discarded as a statistical outlier. A laboratory investigation may be closed out as "inconclusive" only after sufficient work (scientifically sound, and thorough investigation) is performed in an attempt to find an assignable cause for the aberrant/OOS result. It is critical for laboratory management to do due diligence to either confirm the validity of the original result, or to deem the original result invalid or not representative of the product lot tested.

Retesting and Resampling

As part of a laboratory investigation, a company may choose to retest a portion of the original sample, or test a sample retain to assist in determining the cause for the aberrant/OOS result. In most cases, retesting is appropriate when dilution error or equipment malfunction is suspect. Depending on the type of the test performed, different approaches may be needed and should be well defined in the investigation procedure and/or investigation plan, and it often includes the use of a second analyst to verify the suspect result.

The court's decision in the Barr case raised many different opinions on the issue of retesting a product that yields an OOS result. There was an agreement that when laboratory investigation is inconclusive and cannot explain the OOS result, the acceptability of the product batch in question becomes a matter of scientific judg-

ment. Also agreed and enforced by regulators is the need for a company to have a predetermined retesting procedure or plan to address the point at which testing ends and the product lot is rejected, because the goal of retesting is to isolate the aberrant value and not to test a product into compliance.

For most cases, the following guidelines apply when retesting or resampling is required:

- Any retest or resampling must be approved by management and quality assurance.
- A retest should be performed using the same homogeneous material that yielded the anomalous or OOS result.
- Retesting may be done on a second aliquot from the same portion of the sample that was the source of the first aliquot.
- Resampling is performed using a new test specimen.
- If resampling is needed due to a destructive test, the result generated must be annotated as such on the final report.

For microbiological analysis, retesting the original sample may not be possible due to the time lapse between sample collection and when the test result becomes available. In such cases, the company may choose to collect a resample (a new sample) to investigate the anomalous result.

Testing for Outliers

As pointed out earlier in this chapter, statistical calculations for evaluation of outlier results are recommended for microbiological assays, and example calculations are listed in the USP Chapter <111>, *Design and Analysis of Biological Assays*, section *Rejection of Outlying or Aberrant Observations*. According to the USP, an aberrant response or an outlier value may be verified against a criterion based on the variation within a single group of supposedly equivalent responses in a normal population. Calculated gaps equal to or larger than the values listed for G_1, G_2, and G_3 in Table 11.1 (adapted from the USP Chapter <111>, Table 1) occur with probability $P = 0.02$

TABLE 11.1
Test for Outliers—Gap Values

	Critical Values (Gap)										
N	3	4	5	6	7						
G_1	0.976	0.846	0.729	0.644	0.586						
N	8	9	10	11	12	13					
G_2	0.780	0.725	0.678	0.638	0.605	0.578					
N	14	15	16	17	18	19	20	21	22	23	24
G_3	0.602	0.579	0.559	0.542	0.527	0.514	0.502	0.491	0.481	0.472	0.464

Source: Adapted from USP Chapter <111>, Table 1.

where outliers can occur only at one end, or with $P = 0.04$ where they may occur at either end. This criterion is applicable when analyzing, for example, microbial results where each treatment is represented by a value in each of two replicate sets.

USP <111> Statistical Calculation for Outlier Value

1. Beginning with the aberrant value Y_1, list all the results in order of magnitude from Y to Y_n, where n is the total number of results (data points).
2. Calculate the relative gap (G) as follows:

Number of Data Points	Calculation
3–7	$(Y_2 - Y_1)/(Y_n - Y_1) = G_1$
8–13	$(Y_3 - Y_1)/(Y_{n-1} - Y_1) = G_2$
14–24	$(Y_3 - Y_1)/(Y_{n-2} - Y_1) = G_3$

3. If G_1, G_2, or G_3 exceeds the gap-critical value listed in Table 11.1 for the given number of data points (N), there is a statistical basis for omitting the value as an outlier.

The following is an example calculation for the USP outlier test presented.

1. Data set (CFU): 35, 20, 20, 90, 15, 30, 29, 19, 34.
2. Evaluate whether 90 is an outlier value by ranking the values in order of magnitude starting with the putative outlier value:

$$90, 35, 34, 30, 29, 20, 20, 19, 15\ (Y_1 \ldots Y_n).$$

3. As there are nine values, use the following equation to calculate G_2:

$$G_2 = (Y_3 - Y_1)/(Y_{n-1} - Y_1) = (34 - 90)/(19 - 90) = -60/-71 = 0.845$$

4. From Table 11.1, the G_2 value for a set of nine data points is 0.725.
5. Because the calculated G_2 value of 0.845 is greater than 0.725, the result value of 90 CFU can be deemed a statistical outlier.

Repeat Testing

Repeat testing is performed when the outcome of a laboratory investigation proves conclusively that the original test was compromised and is therefore invalid. Example situations that may lead to an invalid test include analyst error, improper sample collection, lack of procedural compliance, equipment malfunctioning, and/or test control failures. Under these circumstances, the original test can be invalidated and the repeat value reported as the official test result. OOS data caused by process- and production-related errors or production equipment malfunction cannot be invalidated, and therefore repeat testing is not allowed. These events represent true product failures, and additional testing would not ensure the quality of the batch in question.

One of the main challenges when conducting an investigation into an anomalous/OOS bioburden or microbial limit test result reside with the fact that microbial contamination is not uniform. Therefore, retests or repeat testing for a microbial limit or bioburden test should be performed to evaluate the overall microbial quality of the product batch in question and not to invalidate the original test result, unless there is conclusive evidence of laboratory error during testing.

Concluding the Investigation

All data generated during laboratory investigation must be compiled in a report, which is then filed in a centralized location. The report must include a conclusion section with identified (or probable) root cause and recommendations (if applicable) for preventative and corrective action (CAPA) to be taken to prevent recurrence of laboratory or system errors.

When conducting a laboratory investigation for an anomalous or OOS microbial result, the typical outcomes are as follows:

- Laboratory error: If there is conclusive evidence of laboratory error, the repeat result substitutes for the original test result, which is deemed invalid.
- Assay variability: If retesting confirms that the OOS result is due to assay variability, all test results, both passing and suspect, should be reported (unless the OOS value is deemed a statistical outlier). In this case, reporting the average of all test results would be an acceptable practice.
- Product failure: If the OOS result is confirmed via retesting, the company must report the result, expand the investigation, and consider the event a manufacturing deviation.
- Inconclusive result: If retesting cannot confirm or discredit the OOS result, the original result cannot be invalidated or discarded. All test results, both passing and suspect, should be reported and considered during batch release decisions.

Troubleshooting microbial anomalous and OOS results can be very challenging, and in many cases the outcome of investigation is inconclusive. Indeed, laboratory investigation for microbiological testing consists of more than retests; it must include a step-by-step evaluation of the entire manufacturing process, the analysis of all evidence, and, if appropriate, performance of alternate tests for confirmatory purposes or to produce additional investigational data.

Laboratory investigations should be trended and trends reviewed on a periodic basis. Assignable causes can be categorized (e.g., analyst error, equipment malfunction, media contamination, etc.) to assist management with evaluation of trends that may reflect problem areas in the testing laboratory or with a manufacturing process. Trending deviations is also a regulatory expectation to ensure CAPA effectiveness; if multiple deviations continue to occur for the same reason, it can be an indication that the CAPA implemented was not effective and it did not address the true root cause of the problem.

Product Lot Disposition

Once laboratory investigation is complete, good scientific judgment must play a role in the decision for batch disposition/release. Companies cannot rely solely on retesting and resampling to release a product lot that failed to meet test specifications unless the investigation conclusively proves that the original sample was compromised during sampling or testing, or that it is not representative of the product batch in question. During this evaluation process, the context of all data generated as well as the product historical data must be taken into consideration for an appropriate decision on batch disposition. Neither the regulatory agencies nor courts (as in the Barr case) will define procedures for product release; such decisions can be made only by the product manufacturer. However, regulators enforce the fact that compendial standards and company-approved product specifications be absolute, not stretched. It is the firm's responsibility to evaluate all quality control data generated and make a final decision as to release of product batch based on supporting documentation that ensures that the material is safe, pure, and effective.

OOS INVESTIGATIONS AND FDA CITATIONS

The recent trend with FDA 483s issued to pharmaceutical companies indicates that "failure investigations" is still one of the top reasons for companies being found noncomplaint with regulations. Presented here is a list of selected FDA 483 citations observed in 2007 and which are associated with laboratory investigations.

1. *BioQuality,* Volume 12(8), August 2007
   - Investigations of deviations were not adequate and completed within established timeframes.
   - OOS result was invalidated and passing retest results used, but no testing was conducted to verify the conclusion that a dilution error had occurred.
   - Quality assurance lacks authority to fully investigate errors that have occurred; for example, lots with multiple deviations, some major, are routinely released without adequate investigation/justification.
   - Environmental monitoring excursion investigations are incomplete and inadequate: organisms are not always identified.
2. *BioQuality,* Volume 12(7), July 2007
   - Nonconformance investigations are not conducted for out-of-specification water for injection (WFI) excursions.
3. *BioQuality,* Volume 12(6), June 2007
   - Investigation into mold contamination of trays was inadequate: production room and equipment were not microbiologically evaluated to determine the type and extent of contamination prior to routine cleaning; no documentation of rationale for decision not to clean with a sporicide as recommended by the microbiology department.
   - Deficient examination into viable particulate alert/action level excursion in compounding room: failed to identify the organisms isolated; corrective and preventative actions were limited to cleaning.

- No investigation into potential source of microorganisms identified during media fill.

4. *BioQuality,* Volume 12(5), May 2007
   - Firm fails to fully investigate the failure of a batch or any of its components whether or not the batch has been distributed.
   - Investigations into deviations and change requests not completed in a timely manner, and no written justification for the delay was available.
   - Procedures addressing verification of corrective/preventative actions not defined and implemented: corrective action reports describe actions taken but lack verification that these actions were effective in correcting the identified problems.
5. *BioQuality,* Volume 12(3), March 2007
   - Firm's deviation investigation procedures are deficient: investigations can be opened and closed by production personnel without review and approval of quality assurance.
   - Discrepancy and failure investigations do not extend to other drug products that may have been associated with the specific failure or discrepancy.
   - Written records of investigation are deficient in that they do not include conclusions and follow-up.
   - Inadequate investigation of microbial action level excursions for WFI system: no evaluation of dead legs in the system as the possible root cause; no effective preventative and corrective actions have been taken.
   - Risk assessment performed as part of the investigation is inadequate: did not include impact on end user.
6. *BioQuality,* Volume 12(2), February 2007
   - Inadequate handling of viable particle action limits that were exceeded during aseptic filling: corrective and preventative actions did not include advising/counseling/retraining operators involved in these excursions.

The author hopes that the information provided in this chapter will help quality assurance and quality control personnel create and revise appropriate documents and company practices so they can prevent similar regulatory observations at their site of business.

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2. ICH Q7 (2001), FDA Guidance for Industry, Q7 Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), August 2001, ICH, Rockville, MD.

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