Rapid Microbiological Methods in the Pharmaceutical Industry

Edited by
Martin C. Easter

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Preface

FITNESS FOR PURPOSE? TIME FOR A REVOLUTION

This book aims to bring together the expert opinion and experience of implementing microbiological methods and their application in the pharmaceutical industry. It has been a long time in preparation but I believe that it will be worth the wait. I am indebted to all the contributors for their persistence and patience.

The stimulus for the creation of this book was a rash of interest in alternative and rapid methods that offered enhanced detection capabilities. However, these methods raised a number of questions, such as how do we validate new methods, would they be accepted by the pharmacopoeias, and, most important, how would the regulators respond?

We will show that significant progress has been has been made toward answering these questions. In fact, there are no real barriers to the introduction of alternative microbiological methods. The only obstacle is fear of change and procrastination.

The biggest challenge is not the technical problems of demonstrating equivalence of a new precise detection method over the inaccuracy of the established cultural methods that are more art than science. Rather, it is to determine the true analytical needs based on sound risk assessment of modern day hazards associated with current high standards in processing equipment, facilities, and good manufacturing practice. Only then will we get appropriate methods and standards that are fit for the purpose.

It is my fervent hope that this book will provide stimulation, inspiration, and confidence to those responsible for ensuring the microbiological safety of pharmaceutical products, so that they will challenge the current methodology with better solutions.

Martin C.Easter
January 2003

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Introduction

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The purpose and intent of this book are to

a) Educate, by providing details about a range of rapid microbiological methods (existing and novel), about their applications, including practical tips, and about their status regarding validation, established use, and regulatory acceptance;
b) Stimulate, by raising awareness of the origin of methods and current issues facing the requirements of microbiology and its associated test methods; and
c) Challenge the reader, practitioners, suppliers, and regulators to seek better, more pragmatic methods for the assessment of microbial hazards and risks, to ensure product and consumer safety.

It is hoped that the excitement and value offered by new technology will be matched by a realistic understanding of the inherent difficulties posed by microbiology, so that common sense and determination may help us to break through the barriers of procrastination and inertia that prevent progress. As Walter D.Wintle has said,

> If you think that you are beaten, you are;
> If you think you dare not, you don't;
> If you'd like to win, but think that you can't,
> It s almost a cinch you won't.
> If you think that you'll lose, you're lost,
> For out in the world we find
> Success begins with a fellow's will;
> It's all in the state of mind.

We should all be collectively motivated to find the alternative way forward such that the methods we use are "fit for their intended purpose": a key feature of the quality management systems that have become a prerequisite for business. The elephant analogy presented by James Belasco (1990) in his book on empowering change within organizations seems appropriate to describe the current situation in pharmaceutical microbiology and its dependence on compendial methods. The analogy describes the use of heavy chains and shackles during the training of elephants to restrict the movements. In later life this conditioning is achieved with only a small metal bracelet around the foot—attached to nothing!

Reliance on the past is no longer an option if we are to gain the improvements we need for the future. This quote from Franklin D.Roosevelt could easily apply to pharmaceutical microbiology: "It is common sense to take a method and try it; if it fails, admit it frankly and try another. But above all, try something. The millions who are in want will not stand by silently forever while the things to satisfy their needs are within easy reach."

MARKET SIZE

In the late 1990s, it was estimated that the global industrial microbiology testing market was almost 1 billion tests per annum, valued at almost $2 billion. The pharmaceutical industry had the fastest growth rate, but it is still second to the food industry in volume and value. The approximately 250 million tests conducted per annum by the pharmaceutical industry represent a value

of almost $650 million, split almost equally among Europe, the United States, and the rest of the world. European markets appear to be the leaders in evaluating and implementing rapid methods. Convenience and ease-of-use tests have been most widely implemented.

More than three-quarters of all current tests are general, nonspecific tests for total bacterial populations; the remainder is largely targeted at indicator organisms, such as coliforms, and yeasts and molds. Only a few percent of all tests are done to detect specific organisms. Final product testing accounts for at least 40% of all samples, similar to the number of tests conducted on in-process samples. Raw material tests comprise less than 15% of all test samples.

Accordingly, there is significant market value and potential for suppliers of alternative and rapid methods. However, relatively few suppliers are prepared and have succeeded in placing alternative technologies on the market. Perhaps this is a result of such factors as the over-zealous search to fulfill every last facet of validation to satisfy a perceived regulatory demand. This approach often leads to excessive documentation of frequently irrelevant aspects of a new method, thus creating an excessive, costly burden on the supplier and enormous delays in getting alternative methods accepted. All of these factors, together with the very conservative nature of the industry and a reluctance to change, conspire against the widespread adoption of alternative microbiological methods.

ISSUES AND CHALLENGES

Microbes and the Analytical Challenge

Reference is frequently made to the fact that most of the methods in use today are more than 100 years old (their historical development is described in a chapter of this book). Our traditional culture methods were designed by the pioneers of our industry for a unique set of circumstances— i.e., to isolate disease-causing agents and to diagnose and characterize the condition. From the information gleaned, an understanding of the disease condition was formed from which subsequent treatment strategies could be formulated and tested. Under these circumstances the microflora were generally present in high numbers and almost in pure culture. Contrast this with the requirement of microbiological methods today, and we see that the task is quite different— i.e., the level of contamination is very, very low, and the primary target is total bioburden, or organisms indicative of poor hygiene and/or environmental cross-contamination or "objectional organisms" (those having the potential to cause harm). This latter term is very poorly understood and open to widely differing interpretation. Objective hazard and risk assessment is required to define a rational test requirement, if any (see below).

What Are We Really Trying to Detect?

Microbes are living dynamic entities that can either increase or decrease in number depending on how they are treated and stored. They have variable forms and metabolic functions that respond differently to different environmental stimuli, including the growth media in which we try to culture them. It is estimated that we know of and know how to culture only 0.01% of microbes on the planet. Compared to a chemical analyte, many vegetative microbes are poorly defined, highly unstable, and unpredictable. Jarvis (1989) described numerous statistical methods that can be applied to the analysis of microbiological data, together with a calculation of error associated with the different stages of microbiological methods. A new technique, called Measurement Uncertainty, has been used by chemists to describe and quantify the error potential of test results, and some microbiologists are also beginning to look at the technique (Jewel 2001; Voysey and Jewell 1999).

The reality of very low bioburdens needs to be carefully considered. A bacterium weighs approximately 10^{-13}g . A bioburden of <10 microbes per gram measured using a typical plate count method of a Microbial Limits Test (MLT) is equivalent to trying to detect 1 part in 10^{12}. In water samples in which a bioburden of 1–10 microbes per 100 ml can be expected, the equivalent analytical challenge is to detect 1 part in 10^{15}. This is a very difficult challenge compared to that facing an analytical chemist who may be looking for impurities in the parts per million or parts per billion range (i.e., one part in 10^6 or 10^9).

Plate counts determined by the MLT are recognized to have a large standard error (up to 71–100%; Anon, 1996), particularly when there are few organisms present (one or two colonies per plate). The MLT on over-the-counter (OTC) products typically yields results that show that on 95% of occasions there is an absence of colonies, which means that a test based on enumeration gives a meaningless result. A more pragmatic approach would be to adopt a presence or absence test (see RapiScreen test in the chapter by Wills on ATP [adenosine triphosphate] bioluminescence).

Test results from microbiological enumeration on water samples with very low bioburdens are equally variable. Enumeration under these circumstances is totally meaningless; trend analysis provides much more valuable and reliable information for decision-making (Wills et al., 1998). The value of data from enumeration techniques in environments with very low bioburden, such as clean rooms, has been questioned by Cundell et al. (1998), who could not demonstrate any

correlation between sampling time and frequency of sampling despite intensive sampling and testing. This begs the question—are we wasting our time, or is there a better way of doing it?

The inherent problem has less to do with the test methodology and more to do with sample distribution error. The distribution of microbes in the test consignment is largely dependent on their point of origin, morphology, and distribution within the lot. The confidence in the test results of a microbiological test can be markedly improved simply by changing the method of sample collection to obtain a more representative aliquot sample for testing. Several strategies have been developed to reduce the impact of distribution error, but these are heavily influenced by the nature of the sample matrix itself. Habraken et al. (1986) showed how the application of a continuous, on-line industrial sampling device was used to improve the statistical confidence of tests to detect the presence of *salmonellae* in milk powder. Confidence in the test result can be increased from <7% to >70% simply by changing the sampling procedures. The application of similar sampling strategies and a realistic assessment of microbiological results would provide assessment techniques that would serve the industry better than do current approaches.

Despite all of the above, it is the expectation and belief of many microbiologists and regulators alike that the traditional cultural methods are the gold standard that give an absolute, reliable enumeration of the microflora present. Traditional cultural methods do not and cannot deliver such information. The results from such methods are used to draw conclusions about product quality and safety often without considering the inherent nature of the product, source of raw materials, and processing parameters and controls. It is time that the inherent weaknesses of our traditional methods were fully recognized and acknowledged, so that we can move on to more meaningful approaches and test methods to assure quality and safety.

Hazard Analysis and Risk Assessment

Is the pharmaceutical industry so different that it cannot learn anything from other industry sectors? No, it is not; it can learn much from the food sector.

Legislation is common. The U.S. Federal Food, Drug, and Cosmetic Act, for instance, has a wide scope, even though different regulatory agencies or departments have responsibility for specific sectors. Its purpose is to ensure consumer protection, and FDA safety and regulatory inspectors have to enforce a wide range of other regulations as well. They cannot be expected to be expert in every single aspect; only approximately 20% have had some formal microbiological training.

Pharmaceutical products are inherently safer than foods. The natural hazards associated with their formulation are fewer, although the risks at the point of use of some (e.g., inhalants and injectables) may be greater. Ingredients and products have a lower bioburden and are less perishable (or are more hostile environments) than foodstuffs, and pharmaceutical and healthcare products are highly processed in very clean manufacturing facilities. There have been few or no incidents of actual harm to consumers arising from microbial contamination of pharmaceutical products; in contrast, every year in the United States alone there are 9,000 deaths associated with the consumption of contaminated foods. This circumstance has resulted in the global introduction and adoption of a safety assurance program based on the principles of hazard analysis and risk assessment. The food safety system, Hazard Analysis Critical Control Point (HACCP), is now included in national and international regulations. This system focuses on the microbiological hazards, their sources, likelihood of occurrence, and methods for prevention, monitoring, and corrective action.

There is no shortage of systems and procedures for hazard analysis and risk assessment in the pharmaceutical industry. Gillet (1996) lists many different techniques (see Table 1.1), but most of them are focused on the chemical processes and environmental hazards. He describes a useful distinction between the intentional and unintentional use of microbes in the industry and reference to HACCP and Failure Modes and Effects Analysis (FMEA), the system from which HACCP was developed. The principle of FMEA with a quantitative assessment of risk will identify microbiological hazards and produce an objective assessment of risk. From this

Table 1.1 Hazard Identification Methods Suitable for Use in the Pharmaceutical Industry

Project phase	Hazard identification Method
Research & Development	"What if?" Checklist Chemical and Operational Hazards Assessments
Feasibility Studies	Preliminary Hazard Analysis "What ifs" MHC MIC Hazard Indices Checklist
Project Definition	Checklist

Project phase	Hazard identification Method
	Threats and Control Analysis Hazard Analysis Critical Control Point (HACCPs)
Design	Failure Modes and Effects Analysis (FMEA) HAZOP HACCP GENHAZ "Sneak" Analysis Fault Tree Analysis
Procurement and Construction	Checklist Process Safety Review
Commissioning	Checklist Process Safety Review
Production	Checklist Process Safety Review SHE audits
Incident Investigation	FMEA Root Cause Analysis "Sneak" Analysis Management Oversight and Risk Tree (MORT) Human Reliability Analysis (HRA) Task Analysis
Modifications	Checklist HAZOP

From Gillett (1996). Reprinted with permission from Interpharm Press.

assessment, it is possible to specify the attributes and requirements of the analytical methods required. Løvtrup (2001) describes the use of Design and Barrier Assessment for risk assessment in the manufacture of medicinals, and Jahnke (1997) applies the seven principles of HACCP. Both recognize that bioburden is a hazard, but the risk is the entry of bioburden during processing to contaminate the product. This contamination is the real issue, not the mere presence of bioburden. More emphasis and attention needs to be directed specifically toward the microbiological hazards within risk assessment techniques to obtain the most benefit.

The pharmaceutical industry needs to apply an effective system to assess the real microbiological hazards and hence quantify realistic risks. Only then will it determine sensible standards and be able to set monitoring methods that will deliver meaningful, useful data for effective decision making in manufacturing and quality assurance, to ensure product safety. A commonsense approach is required that can put the perceived microbiological hazards and risks into perspective and can lead to development of new strategies for process control that rely more on physical parameters and less on microbiological assessment (Akers 2001).

HOPES FOR THE FUTURE

I believe that we are at a watershed in pharmaceutical microbiology and that we need a very pragmatic approach, a clear vision of intent, and the determination to make change happen.

Many existing microbiological methods have been validated, but few have found widespread application in the pharmaceutical industry. Several new techniques offer huge potential for the future but need to be evaluated prior to validation. The issue is not the availability of potential methods but a reconciliation of our requirements, the recognition of the limits of current methods (including sampling statistics), and what can actually be achieved in practice.

Many forward-thinking people are able to comprehend the task, but we need to create the correct culture, framework, and environment to facilitate the change. Technology users, suppliers, and regulators have to be included. Such an initiative under the Parenteral Drug Association (PDA) has resulted in the publication of a specific technical report for the evaluation and validation of microbiological methods (see Bauer et al. 2000). Meeting validation requirements is a relatively new task that microbiologists often fear unnecessarily because of the uncertainty factor. Analytical chemists frequently develop and validate chemical methods for new drugs and products without difficulty. There are no barriers to alternative methods; in fact, regulators want to see the use of best practice and do not want to impede innovation.

The industry will get suitable meaningful methods only when it conducts an objective assessment of microbiological hazards and risks, chooses technologies that are fit for their intended purpose and can meet the analytical requirements, and challenges the *status quo* to embrace change for the better. The pharmaceutical industry has to rise to the challenge.

Charles Darwin said that "it is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change."

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2
Historical Perspective on Methods Development

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> "C'est un grand progrès, Monsieur." Louis Pasteur to Robert Koch on observing his demonstration of the plate technique at the Seventh International Medical Congress, London, August 1881.

If the pioneering German bacteriologist Robert Koch visited a microbiological testing laboratory in the pharmaceutical, biotechnology, or medical device industry today, he would recognize that most of our techniques were first developed or used in his laboratory at the Imperial Health Office and then later at the Institute for Infectious Diseases in the German imperial capital of Berlin during the last three decades of the nineteenth century. These methods include the fixing and staining of bacterial cells on glass slides for microscopic examination and photomicroscopy, growth of colonies on solid media, streaking for isolation of pure cultures on solid media, the use of agar-agar as a support for microbiological media in Petri dishes, serial dilution and plating on solid media to enumerate the microbial population in water, monitoring bacteria within the air, the classification of bacteria by their cellular morphology and differential staining, sterilization of microbiological media by filtration or steam sterilization, disinfectant testing, and aerobic and anaerobic incubation. Ironically, the very success of these classical pure culture techniques in elucidating the role of microorganisms as infectious agents of disease has led to a general conservatism in the implementation of new microbiological methods in routine clinical, food, and pharmaceutical microbiological testing laboratories.

In this survey of the historic development of microbiological techniques, emphasis is given to the introduction of new methods, concepts, and technologies, and the publication of standard methods as background to the more detailed discussion, in subsequent chapters of this book, of the application and acceptance of rapid microbiological methods in the pharmaceutical industry.

FOUNDATION OF THE FIELD OF MICROBIOLOGY

From his earlier research in crystallography, where he established the relationship between optical activity and crystalline asymmetry, French chemist Louis Pasteur turned to the study of fermentation and spontaneous generation. In a series of brilliant experiments before the French Academy of Science, Pasteur was able to demonstrate that fermenting microorganisms came from the surrounding air and not from spontaneous generation. Boiling the solutions and heating the air excluded bacteria, and spontaneous generation of ferments in milk and sugar solutions did not occur as was widely believed by his contemporaries.

From this work by Pasteur came the foundation of the scientific field of microbiology and the practical applications of the pasteurization of milk, beer, and wine, aseptic surgery, and the treatment of infectious disease. Although Pasteur was the founder of modern microbiology, he did not succeed in the routine isolation of pure cultures, as we know it, necessary to elucidate the organisms responsible for individual diseases. This achievement belongs to his German rival, Robert Koch. However, Pasteur' s work established the scientific basis of vaccination, and his practical interests led to the application of microbiology to industrial processes. Pasteur made the critical step from his work on fermentation and disproof of spontaneous generation to the germ theory of putrefaction and disease, which inspired the English surgeon Joseph Lister, who introduced aseptic surgery. After much debate, scientists and physicians accepted that microorganisms were the primary cause of infection, and Pasteur's findings paved the way for microbiology as a scientific discipline and revolutionized medicine.

This acceptance was summed up in a letter Lister sent to Pasteur in 1874, in which he said "Your brilliant researches demonstrated the truth of the germ theory of putrefaction and thus furnished me with the principle upon which alone the aseptic system can be carried out."

The research undertaken by Pasteur on the spoilage of beer may be considered the first systematic activities of an industrial microbiologist and should stand as a model to us on the control of microbial contamination in the manufacturing environment and our products. Even today, the 1879 English translation of this research, which was undertaken in a spirit of nationalism to help the French breweries challenge the German beer industry, makes interesting reading.

DEVELOPMENT OF THE CLASSICAL MICROBIOLOGICAL METHODS

In 1872 the German botanist Oscar Brefeld reported growing fungal colonies from single spores on gelatin surfaces. Prior to this innovation, which resulted in the isolation of pure culture of microorganisms, pigmented bacterial colonies were isolated by the German biologist Joseph Schroeter on slices of potato incubated in a moist environment. In his groundbreaking study of the natural history of anthrax, Robert Koch, while still a country physician, inoculated barnyard mice with diseased tissue from farm animals that had died of anthrax, reproducing the disease in these improvised laboratory animals. The bacterium responsible for anthrax was cultured in ox aqueous humor, and fixed, and stained the culture on a glass slide. Using the newly developed technique of photomicroscopy, Koch captured the image of bacterial cells.

In the summer of 1877, Koch demonstrated the proof that a specific bacterium *Bacillus anthracis* caused a specific disease, anthrax, to the preeminent German microbiologist Ferdinand Cohn. Cohn recognized the importance of the work, helped publish the findings, to clearly establish the scientific basis of the germ theory of disease, and helped Koch find positions suitable for a scientist of his enormous talent.

It was Koch who fully appreciated the value of microscopy in the study of microorganisms. He believed that the discovery of bacteria in diseased tissue was an important first step toward the isolation of the causal agents. Visits to the Zeiss microscope factory in the town of Jena, Germany, enabled him to purchase and use an Abbe condenser to concentrate light on the microscope objective and the oil-immersion objective lens to recognize different bacteria by their morphology and differential staining within diseased tissue.

In July 1880 Koch secured a position in charge of bacteriological research at the Imperial Health Office in Berlin. He was fortunate in obtaining as his first two assistants Friedrich Loeffler, who later isolated *Corynebacterium diphtheriae,* the causal agent of diphtheria, and Georg Gaffky, who isolated the typhoid bacillus and succeeded Koch as the director of the Institute for Infectious Diseases. Koch had the ability to attract and develop a generation of pioneering microbiologists from around the world. Later joining Koch at the Institute of Hygiene, University of Berlin, and the Institute for Infectious Diseases were Richard Pfeiffer, who discovered the immunological lysis of *Vibrio cholera;* Emil von Behring and the Japanese microbiologist Shibasaburo Kitasato, who codiscovered diphtheria and tetanus antitoxin; August Wasserman, who developed the serological test for syphilis; and Paul Ehrlich, who founded chemotherapy. Prominent American microbiologists who were students or intellectual heirs of Koch include William H.Welch, professor of pathology at Johns Hopkins University and the discoverer of the causal agent of gas gangrene, *Clostridium perfringens;* Herman M. Biggs, head of the New York City Department of Health; and T.Mitchell Prudden, professor of pathology at Columbia University.

The period that followed the appointment of Koch as the head of the Institute for Infectious Diseases was the most significant five years in the development of microbiological techniques. Although Pasteur had used serial transfers in broth with microscopic examination to confirm the isolation of pure cultures and Lister had used a specially constructed syringe to recover a one-millionth dilution of a culture of soured milk to isolate a pure culture of *Streptococcus lactis,* it was Koch who established the plate technique to routinely isolate pure cultures of microorganisms.

Essentially Koch took nutrient broth, which unlike the slices of potato used by Cohn's student, Schroeter, in his study of pigmented bacteria, supported the growth of pathogens, and added gelatin to make it solid to isolate pure cultures as distinctive colonies on the plate. This critical improvement in pure culture technique was described by Koch in the seminal paper "Methods for the study of pathogenic organisms" in the first volume of the in-house journal of the Imperial Health Office *Mittheilungen aus dem Kaiserlichen Gesundheitsamte.* Equally important was his demonstration of these new methods to the many visitors to his laboratory. These techniques enabled Koch and his co-workers to determine the types and numbers of microorganisms in air, water, food, and soil, as well as clinical samples.

Two additional steps were needed to give us the familiar solid agar microbiological culture medium in a Petri plate. Gelatin had the disadvantage of liquefying at the higher ranges of room temperature, so that the plates could not be incubated at the above-ambient temperatures that are optimal for many pathogenic bacteria. Furthermore, it was not inert as it was hydrolyzed by proteolytic bacteria. In 1882 Koch's associate Walter Hesse, on the suggestion of his New Jersey-born wife, Fannie Hesse, substituted for gelatin the gelling agent agar-agar, used in Asian cooking, which remained solid at higher incubation temperatures. The flat, indented glass plates that supported the agar or gelatin were replaced by flat double-sided dishes, which did not require a special pouring apparatus developed by Koch and storage in a bell jar. The innovation we call Petri

plates or dishes, after Koch's associate Richard Petri, were sterilized separately by dry heat, and the molten nutrient agar poured into the dish and inoculated. The plates are incubated as stacks of dishes, and the colonies developing within the microbiological medium are examined, counted, or isolated. After the First World War, Petri dishes were made from Pyrex® glass; the glass was replaced by disposable plastic by the late 1950s.

These techniques were revolutionary with respect to the study of microbiology. Their use resulted in the discovery of the causal agent of tuberculosis, *Mycobacterium tuberculosis,* within an eight-month period between August 1881 and March 1882. Koch used differential staining to visualize *M. tuberculosis* from the surrounding lung tissue. Using aseptic techniques, he transferred the organism from humans to guinea pigs, to demonstrate its role in causing the disease. He then isolated the slow-growing bacterium in pure culture on serum agar plates. The discovery of the bacterium that caused tuberculosis was an international sensation, as it resulted in the promise of a cure for tuberculosis.

However, the discovery of a cure for tuberculosis eluded Koch when he unsuccessfully promoted tuberculin as a treatment. Eventually, this failure drove him out of Berlin to travel with his second wife and study tropical diseases, far from the public pressures in Germany following his scientific failures and scandalous divorce. His work on tuberculosis was eventually recognized within the scientific community when he belatedly was awarded the 1905 Nobel Prize for medicine.

Other techniques that were critical to the development of microbiology include dry heat, steam and filter sterilization, above ambient temperature incubation, anaerobic and aerobic incubation, chemical disinfectant, differential staining, microbial classification by morphology, biochemical reactivity, and physiology.

The work of Pasteur in discrediting the concept of spontaneous generation led to his preparation of sterile broth by boiling. In 1861 Pasteur found that acid infusions but not alkaline infusions were sterilized by boiling, but he did not know why. The classic work by the English physicist John Tyndall in 1877 showed that discontinuous heating, now termed tyndallization, was needed to sterilize the hay infusions that we now know contained spore-forming bacteria. The German pioneering microbiologist Ferdinand Cohn discovered the bacterium *Bacillus subtilis* from hay and first attributed the formation of endospores to the resistance of the organism to boiling. Cohn first used the now familiar flask and test tubes of media plugged with sterile cotton wool. The extraordinary sterilizing property of superheated steam was described by Pasteur in the form of the effect of immersing sealed flasks in a bath of calcium chloride solution above 100°C. Pressurized stream kettles became common in the laboratories of the pioneering microbiologists. However, the first practical autoclave as we know it was constructed in 1884 by a Parisian engineering company for Charles Chamberland, a co-worker of Pasteur.

Lister described a dry heat sterilization procedure in his classic 1877 publication on lactic fermentation. In this study he heated his glassware, as we still do, to 350°C for 2 hours in a dry heat oven to sterilize the equipment.

In 1884 Chamberland described the candle-form filter, composed of unglazed porcelain, that was used to sterile filter bacteriological media and tap water. This type of filter was used by the German veterinary microbiologist Paul Frosch to demonstrate that the causal agent, later recognized as a virus, of foot-and-mouth disease could pass through a sterilizing filter.

In 1861 Pasteur introduced the terms *aerobic* and *anaerobic* in describing the growth of yeast at the expense of sugar in the presence or absence of oxygen. He observed that more alcohol was produced in the absence of oxygen when sugar is fermented, which is now termed the Pasteur effect. The first strict anaerobic pathogen, the tetanus bacilli *Clostridium tetani,* was described by Kitasato in 1889.

The first systematic attempt to investigate the culture and temperature requirements of a microorganism was that of the French mycologist Paul Raulin, a student of Pasteur. In 1869 Raulin reported placing *Aspergillus niger* spores in saucers of chemically defined media consisting of water, sugar, and minerals and incubating the mold at temperatures in the range of 20 to 38°C. Raulin constructed incubators with heating coils containing circulated warm water and controlled the humidity at 70% RH to maintain a temperature within 1°C. This led to complaints from the principal of the Ecole Normale on the large consumption of gas to heat the incubators. Apparently, university administrators have always been conscious of overhead costs associated with scientific research.

In 1881 Koch systematically investigated the efficacy of chemical disinfectants, demonstrating that the carbolic acid used by Lister in aseptic surgery was merely bacteriostatic and not bactericidal. He first recognized that disinfection depended on the chemical concentration and contact time. Anthrax spores were dried on silk threads, exposed to disinfectants, washed with sterile water, and cultured to evaluate a range of chemicals. The need to inactivate the disinfectant prior to culture to avoid undercounting the surviving bacteria was discovered by fellow German August Geppert who precipitated mercuric chloride with ammonium sulfide so as not to overestimate the efficacy of the antimicrobial agent mercuric chloride. The field was fully established on a quantitative basis by the Germans Bernard Kronig and Theodor Paul in their classic 1897 publication.

The German pathologist Carl Weigert first used aniline dyes in 1879 to demonstrate the presence of bacteria in tissue. Paul Ehrlich, who would go on to win a Nobel prize for his work in chemotherapy, worked with Koch to improve the staining of the tubercular bacillus, publishing his paper on acid fast staining in 1882. Differential staining of bacteria that is universally used to classify them as negatively or positively reactive to what is now called the Gram's stain was developed from Ehrlich's work by the Danish microbiologist Christian Gram in 1884.

In 1883 Koch lectured to a medical group on the application of plating methods to the systematic study of microorganisms in air, water, and soil. Hesse achieved counting of airborne bacteria by drawing air through tubes lined by gelatin and incubating them so the colonies could be counted. The organisms isolated from the Berlin air, for example, micrococci, bacilli, and aspergilli, are those familiar to us today when we monitor the air in our Class 100,000 pharmaceutical manufacturing areas. Counts of bacteria in water were achieved by adding 1 ml of water to the plate and pouring in the nutrient gelatin. This is the now familiar pour plate method. Similar approaches to counting bacteria in soil revealed that the number of bacteria decreased from the organic-rich upper to the organic-poor lower soil layers.

The pioneer in the classification of bacteria was Cohn, who first suggested the division of bacteria into groups based on their morphology. His belief that bacteria formed distinct genera and species was revolutionary at the time, as the diversity of bacteria was not appreciated. Cohn recognized six genera without the advantages of pure culture and staining techniques. They were *Micrococcus* (ball or egg-shaped), *Bacterium* (short, rod-like), *Bacillus* (straight, fiber-like), *Vibrio* (wavy, curl-like), *Spirillum* (short, screw-like), and *Spirochete* (long, flexible, spiral). The influence of Cohn and Koch can be seen in the later work of the Scottish surgeon Alexander Ogston, who in 1883 differentiated between two micrococci in pus; one, arranged in chains, is now called *Streptococcus,* and, the other, arranged in masses, he called *Staphylococcus.*

The progress that Koch had made with the development of microbiological methods is illustrated by the list of equipment, including culture vessels, media, inoculation equipment, stains, microscopes, surgical instruments, and sterilization apparatus, that the 1883–84 German Cholera Expedition took to Egypt and India. Koch's approach of detecting the pathogen in diseased tissue of the intestinal tract and isolating the causal organism *Vibrio cholera* in pure culture was successful in the Indian leg of his scientific journey.

Koch described his methods as follows:

> Up to now, 22 cholera victims and 17 cholera patients have been examined in Calcutta, with the help of both the microscope and gelatin cultures. In all cases the comma bacillus and only the comma bacillus has been found. These results, taken together with those obtained in Egypt, prove that we have found the pathogen responsible for cholera.

DEVELOPMENT OF ADVANCED MICROBIOLOGICAL METHODS

The concept of selective or enrichment culture was introduced at the beginning of the twentieth century by both the Dutch microbiologist Martinus Beijerinck and the Russian microbiologist Serge Winogradsky. They isolated, from the environment, microorganisms capable of specific physiological or biochemical function; this work had a pronounced effect on the study of the ecological relationships of microorganisms to their environment, the classification of microorganisms based not only their morphology but also their physiology, and the development of differential culture in isolating pathogenic microorganisms from clinical specimens. Influential in the change from classifying bacteria on the basis of their morphology to the use of their physiological characteristics was the 1908 publication of the Danish dairy microbiologist Carl Orla-Jensen. He emphasized the definition of the types of oxidations and fermentations conducted by bacteria in terms of chemical reactions with organic and inorganic compounds and liberation of energy. A practical outgrowth of this approach was the 1912 introduction of the methylene blue reduction test for grading milk.

The established system of bacterial classification based on colony morphology and color, cellular morphology, differential straining, motility, physiology and biochemistry used by American microbiologists is described in *Bergey's Manual of Determinative Bacteriology.* The routine identification methods employed in the microbiology laboratory continue to be based on the determination of the morphology, differential staining, and physiology of a bacterial isolate by the means of miniaturized substrate utilization screening methods to speciate the isolate, e.g., Vitek and Biolog microbial identification systems.

The influential publications in the development of microbiological methods in the United States include Herman M.Biggs's translation of the 1886 pioneering microbiological textbook by the German biologist Ferd70inand Hueppe entitled *Methods of Bacteriological Investigation;* the American Public Health Association first edition of *Standard Methods of Water Analysis* in 1905; American Public Health Association first edition of *Standard Methods of Bacterial Milk Analysis* in 1910; the treatise on pathogenic bacteria published as *Zinsser Microbiology* in 1910; the Association of Official Analytical Chemists (now AOAC International) validation of official methods in 1916; the first publication of the *Journal of Bacteriology* edited by Charles-Edward A. Winslow, professor of public health, Yale University, in 1916; *Bergey's Manual of Determinative Bacteriology* in 1923; the sterility test, which first appeared in the eleventh *U.S. Pharmacopeia* in 1936; the first edition of *FDA Bacteriological Analytical Manual* in 1965; and *Manual of Clinical Microbiology* published by the American Society for Microbiology in 1970. The importance of the publication of standard methods cannot be overemphasized. For example, the current (twentieth) edition of the text *Standard Methods of Water and Waste Water Analysis* contains the details of the pour plate and membrane filtration methods we use to enumerate bacteria in Chapter <1231> Water For Pharmaceutical Purposes as defined by the U.S. Pharmacopeia.

THE SEARCH FOR RAPID MICROBIOLOGICAL METHODS

With the beginning of the twentieth century the impact of microbiology in the areas of public health, agriculture, and medicine was increasing with the developments in immunology and chemotherapy being the most profound. Soon after the classic 1895 work on the microflora of the human intestine by the German microbiologist Theodor Escherich in which he discovered *Bacillus (Escherichia) coli,* it was suggested by his fellow German Max Schardinger that coliform bacteria be used as indicators of fecal contamination of water. Water samples may be analyzed for coliforms by using the most probable number, multiple-tube fermentation test, which is based on the ability of coliforms to grow in selective broth at 35°C, producing acid or gas within 24 to 48 hours. The number of coliforms and the 95% confidence limit can be determined by using Most Probable Number (MPN) tables for the volumes and number of fermentation tubes used (McGrady 1915). Because of the delay in culturing microorganisms, there has historically been an interest in rapid microbiological methods. Examples of the name of principal investigators and the date of their discoveries used to reduce the incubation time to enumerate and identify microorganisms are as follows: serological methods introduction for the rapid identification of the cholera vibrio and the typhoid bacillus by Gruber and Durham in 1896; measurement of bacterial growth by monitoring changes in the electrical conductivity of the medium by Stewart in 1899; microscopic identification of microcolonies used to count bacteria within 4 to 6 hours of incubation by Frost in 1921; and use of a fluorescent antibody technique to detect pathogens like *Salmonella* in food and clinical specimens by Coons in 1940.

The most significant post-Second World War advancement in microbiological methods was the introduction of membrane filtration techniques to count microorganisms. Sartorius-Werke AG developed membrane filters commercially, in Germany. Prior to the war, membrane filters were primarily used for sterile filtration. In a response to the need to determine water quality after wartime bombing, the German Hygiene Institutes used membrane filtration for culturing coliforms. In 1947 the German microbiologist Robert Muller published a method of counting coliforms on membrane filters using a Lactose-Fushsin broth. Today, membrane filtration is widely used for counting microorganisms in beverages, water and wastewater, food, and pharmaceutical products.

The technique is based on passing the sample through a 47 mm diameter membrane filter and entrapping the microorganisms with a 0.45 micron sized pores. The filter is placed on the appropriate medium and incubated. The colonies are counted after 24 to 72 hours. The method is more precise than the MPN multiple-tube technique, but the test sample must be filterable.

The next series of advances came out of NASA-sponsored research to detect life on Mars. The techniques used were (1) ATP bioluminescence, based on the 1944 work of American biochemist James McElroy on American firefly bioluminescence and (2) new advances in gas-liquid chromatography. These technologies have been fully commercialized in the Millipore/ Celsis SteriScreen, the MicroStar Microbial Enumeration System, and MicroCount digital ATP Bioluminescence Systems and the MIDI (gas liquid chromatographic fatty acid analysis) Microbial Identification System.

From the Woods Hole Marine Station summer research program came the discovery that the lysate of the amebocytes from the hemolymph of the horseshoe crab, *Limulus polyphemus* clots in the presence of the lipopolysaccharides in the cell walls of gram-negative bacteria (Levin and Bang 1968). This finding led to the development of an in vitro assay for the pyrogens that contaminated injectable products, which replaced the rabbit pyrogen test. The replacement of an animal test with a more sensitive and convenient Limulus Amebocyte Lysate (LAL) test can be seen as a prototype for the future replacement of culture methods with biochemical methods.

Important milestones in the development of microbiological methods listed by the names of the innovator and the date of the innovation include the use of the API strip for the miniaturization of biochemical tests for bacterial identification by Buissiere in 1972; measurement of bacterial growth using impedance by Cady in 1975; the use of acridine orange to stain marine bacteria collected on a polycarbonate filter which were counted by epifluorescence microscopy by Daley and Hobbie in 1975; from fluorescence-activated cell sorting, flow microfluorometry was developed for the detection of bacteria by Hagenson in 1976; and the use of hydrophobic grids on a membrane filter to create the 1600-tube single MPN test, capable of counting bacteria, fungi, and coliforms over a 4-log range with a reduced incubation time by Sharpe in 1976.

Other technologies that have been developed are based on hydrolytic enzymes that cleave fluorogenic substrates for beta-galactosides, beta-glucuronides, and esters found in target organisms. For example, presence-absence tests for coliforms have been developed from the production of a fluorescent end product when beta-glucuronidase from *E. coli* hydrolyzes the fluorogenic substrate 4-methylumbelliferyl-beta-D-galactoside (MUG). These substrates can be used as viability strains for microorganisms when the fluorogenic substrate diffuses or is transported into a microbial cell, and is hydrolyzed, liberating fluorescein that accumulates sufficiently within the cell to fluoresce when excited by light of the appropriate wavelength. The development of a rapid method to detect single fluorescent microbial cells on a membrane filter in the Chemunex Scan RDI System was the result of combining the technologies of fluorogenic substrates, membrane filtration, laser scanning, and fluorescent microscopy.

In 1955 the chemical structure and the base pairing of the DNA molecule were reported by the American biologist James Watson and English physicist Francis Crick. Their Nobel prizewinning publication in the British scientific journal *Nature* was the beginning of the new field of molecular biology, which would revolutionize both biology and medicine.

Further important milestones identified by name of the discover and the date of the discovery in the development of molecular biology-based microbiological methods are the enzyme DNA polymerase that is responsible for the replication of nucleic acid by Kornberg in 1958; DNA hybridization first used to identify bacteria by Grunstein and Hogness in 1975; first use of ribosomal 16S RNA homology in bacterial taxonomy by Woese in 1977; DNA sequencing by Sanger in 1977; and Polymerase Chain Reaction (PCR) developed to rapidly amplify a DNA segment by Mullis in 1985.

The key to the whole field of nucleic acid-based identification of microorganisms was the introduction of the concept of molecular systematics using proteins and nucleic acids by the American Nobel laureate Linus Pauling in 1965. Since the sequence of nucleic acids in a particular microorganism is an extremely conservative constant, even over geological time, and the DNA and RNA molecules are relatively stable, they are excellent materials for the detection and identification of microorganisms. However, this identification method is complicated by the fact that a single bacterial cell weighs 10^{-13} g, and the nucleic acid sequences that are amplified by PCR may represent 100 to 100,000 times less weight than that of the bacterial cell.

To detect a single *Salmonella* cell in 10 g of a pharmaceutical product, an enrichment culture would be used to obtain at least 10^3 cells. The resulting cells would be lyzed, and a thermostable polymerase used to copy a nucleic acid strand using oligonucleotide primers specific for *Salmonella.* This is achieved by using a sequence of temperature cycles to denature the nucleic acid, hybridize it by the means of primers, and use of polymerase-mediated complementary-strand synthesis to produce more nucleic acid. Twenty to thirty thermocycles can increase the amount of target nucleic acid over one million times so it can be detected by its electrophoretic pattern. This technology has been commercialized for the detection of *Salmonella* spp. as the BAX® PCR Microbial Detection System. With this technology it should be possible to develop a multiplex PCR system for the simultaneous detection of the USP indicator organisms *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Salmonella* spp.

Perhaps more mundane, but no less important, was the development and introduction of a wide range of equipment and utilities that are found in the pharmaceutical microbiology laboratory. The list of the notable developments, name of the developer and date of the introduction includes incubators by Raulin and Pasteur in 1869, refrigerators by von Linde in 1876, dry heat ovens by Lister in 1877, centrifuges by Laval in 1878, autoclaves by Chamberland in 1884, pH meters by Haber in 1909, heat resistant glassware by Sullivan and Taylor in 1915, spectrophotometers by Dreosti in 1931, and HEPA filters by Whitefield in 1961. Specific mention must be given to the development of the microprocessor, an integrated circuit on a silicone chip that was introduced by the Intel Corporation in 1971 and that made the personal computer and smart laboratory instrumentation possible.

In conclusion, a major trend is underway in the pharmaceutical microbiological testing laboratory, where the classical microbiological cultural methods that were developed in the late nineteenth century will be replaced for routine testing by biochemical, fluorescent cytometric, and nucleic acid-based techniques. Although many companies have developed instruments to automate the running or miniaturizing of existing test methods, technological improvements are progressing rapidly with new methods based on fluorescent laser detection and nucleic acidbased detection. The future of microbiological testing will lie with the commercialization of automated specific detection methods that will reduce our reliance on culture methods. This will result in routine testing with significantly shorter test cycle time and great quality of results. If Robert Koch could visit our laboratories within the next five years, will he recognize the equipment and testing methods that we routinely use in the microbiological testing laboratory?

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3
Microbiological Methods of the Pharmacopoeia
Growth and Recovery of Microorganisms from Pharmaceutical Manufacturing Environments

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MICROBIAL GROWTH IN PHARMACEUTICAL ENVIRONMENTS: STARVATION-SURVIVAL

The ability of bacteria to act as self-replicating entities distinguishes these contaminants from other, abiological, particulates. Their growth, replication, and production of various ionic and organic by-products in otherwise contaminant-free purified waters, clean-room air, and manufacturing surfaces presents a tremendous challenge to production and quality assurance personnel. The same metabolic and structural properties that enable survival in such an otherwise hostile environment create special problems for bacterial detection and treatment.

Virtually all bacteria isolated from purified water systems are gram-negative, nonfermentative bacilli. The gram-negative cell wall consists of multiple layers of phospholipids surrounded by a lipid-polysaccharide structure (LPS). This multi-laminate structure may afford the cell protection from the extremely hypotonic environment that is intrinsic to purified water systems. These bacteria are heterotrophic, requiring the presence of reduced organic compounds as energy sources. Therefore, these compounds serve as limiting growth factors in purified water systems.

A variety of bacterial genera have been recovered from air and surfaces in manufacturing environments. As with purified waters, it is the nature of the suspending milieu that determines the microbial ecology of air and surface environments. For example, *Bacillus* spp., along with some *Micrococcus* spp., is well adapted to survival in low-water-activity environments (Andersson et al. 1997; Heidelberg et al. 1997). Perfettini et al. (1990) showed that cemented surfaces could provide an environment for fungal growth. Fungi are more commonly isolated from nonaqueous environments such as air handling systems and production environments. The ability of some bacteria and fungi to fix atmospheric CO_2 and NO_x compounds into cell mass is another feature of surface-associated microorganisms.

When one or more essential growth factors is limited, as is often the case in benthic environments and purified water systems, bacteria utilize several strategies designed to assure their survival. The term *oligotroph* has been assigned to organisms that are capable of growth in media containing <1 mg/L organic carbon (Tabor et al. 1981; Ishida and Kadota 1981). In general, a positive correlation exists between assimilable organic carbon levels and planktonic bacterial numbers in purified waters. In the short term, "starved" bacteria tend to use endogenous energy reserves for replication; therefore, one strategy for survival involves replication to increase the probability of species survival when additional energy sources become available (Novitsky and Morita 1978). In marine systems limited in assimilable nutrients, some bacteria reduce their cell volume, forming "ultramicrobacteria" (Morita 1985). Tabor et al. (1981) demonstrated that these organisms, many of which were <0.3 μm in diameter, were capable of passing through 0.45 μm pore-sized microporous membrane filters. Their findings tend to corroborate the work of Christian and Meltzer (1986) and others regarding bacterial penetration of sterilizing-grade microporous membrane filters. Whether this same type of strategy is utilized in nonaqueous environments is unknown at this time.

Population densities in purified waters have been reported to exceed 10^6 CFU (colony forming units)/mL (Carson et al. 1973; Mittelman 1995). McFeters et al. (1993) found 10^2 to 10^3 CFU/mL in a model laboratory water system with many of the same system components (deionization beds, UV, and microporous membrane filters) seen in pharmaceutical-grade purified water systems. As with a number of other workers (Mittelman et al. 1987; Patterson et al. 1991; Martyak et al. 1993), this group found that significantly greater numbers of bacteria (up to 10^5 CFU/cm^2) were associated with various surfaces within the water production and distribution systems.

BIOFILM FORMATION

Perhaps the most significant adaptive mechanism used by bacteria is adhesion to surfaces; indeed, the majority of bacteria in nutrient-limited environments are attached to surfaces. Recognition of this important growth characteristic is a key consideration in developing effective monitoring programs. Sampling of planktonic environments, whether by membrane filtration or the application of evolving electrochemical and acoustic techniques, can recover only a small fraction of the total system bioburden. Several workers have shown that nutrient-limiting environments promote the attachment of bacteria to surfaces (Mittelman et al. 1987; Marshall 1988). Surface area is a major limiting factor for microbial growth in nearly every environment. The ratio of planktonic (free-floating) bacteria to biofilm bacteria is a function of several interrelated factors, including surface energetics, materials of construction (Lewis and Gilmour 1987; Vanhaecke et al. 1990), topography, hydraulic factors, and biofilm chemistry.

Adhesion may be defined as the discrete association between a bacterium and a surface. If a bacterium has adhered to a surface, energy is required to effect a separation. At the molecular level, bacteria adhere to surfaces by a combination of electrostatic and hydrophobic interactions. The nature of these interactions is dependent upon a combination of physicochemical factors, including pH, temperature, ionic strength, ligand density, dipole moment, and charge density (Mittelman et al. 1998). The size and charge of bacteria are in the same range as colloidal solutes. Therefore bacteria can exist as lyophobic or lyophilic sols, association colloids, gels, or part of an emulsion.

The theory of colloid stabilization as applied by Marshall (1988) and others holds that the separation between bacteria and adsorbents in an electrolyte solution is dependent upon a balance between attractive and repulsive forces. The repulsive forces, mediated by like charges on both the bacterial and substratum surfaces, are balanced by attractive forces, which include the hydrophobic effect and hydrogen bonding. This relationship can be described by the following equation:

$$Vt = Vr + Va,$$

where Vt=total energy of interactions, Vr=energy of repulsion, and Va=energy of attraction.

INFLUENCE OF ENVIRONMENTAL CONDITIONS ON MICROBIAL SURVIVAL

Bacteria possess adaptive mechanisms for responding to those physicochemical factors that define their environment. These factors include nutrient availability, pH, Eh, temperature, organic and ionic content, and the presence of antagonistic agents. Depending upon the types and numbers present, bacteria can effect alterations in their physiology or physical state in response to the environment. By extension, these factors are important considerations in the development of effective sampling, enumeration, and identification techniques.

The pH of aqueous and nonaqueous environments influences cell surface charges and thus the cell's adhesion to surfaces, as well as the binding capacity of the cell surface (Herald and Zottola 1987; Levy 1987). Additionally, pH governs extracellular polymer stability. The chief consideration of pH may be the negative effects of pH values below 6 upon organism survival or culturability using classical techniques. Ionic strength is another major determinant of cell surface charge and, by extension, the association of bacteria with charged surfaces. Fletcher (1988) showed, for example, that the adhesion of *Pseudomonas fluorescens* to glass substrata was favored under conditions of increased ionic strength (addition of NaCl).

The availability of assimilable organic carbon is a key determinant of both cell size (Morita 1985; Nystrom et al. 1992) and cell-surface interactions (Dahlback et al. 1981; Wrangstadh et al. 1986). As nutrient levels decrease, cells increase their ratio of cell surface: volume and export hydrophobic proteins to the cell wall exterior (Novitsky and Morita 1978; Nystrom et al. 1992). The net effect of these two actions is to miniaturize cell size and increase hydrophobic interactions with substrata, respectively. Several workers have shown that nutrient-limiting environments promote the attachment of bacteria to surfaces (Marshall et al. 1971; Mittelman and White 1990). For example, Davies and McFeters (1988) found that planktonic (free-floating) bacterial growth on low-nutrient minimal media resulted in a significant reduction in bacterial size in a simulated drinking water environment. Predictions of whether a given organism will adhere to a particular surface are highly dependent upon the nature of the suspending host milieu and the physiological status of the bacterial community present. Bacterial metabolism and cell-surface structures are influenced by the physicochemical characteristics (e.g., surface charge, functional groups, steric factors) of the substratum (Mittelman 1998).

Bacteria surviving in pharmaceutical environments must adapt to survival under relatively extreme environmental conditions of low water activity (surfaces; some products), high or low temperatures (water systems; equipment), and extremely low nutrient concentrations (water systems; some products). These same organisms, when cultured under eutrophic and mesothermic conditions, may fail to grow and produce visible colonies. Therefore, recovery and enumeration techniques should take into account the environmental conditions under which the organisms were recovered. The development of the low-carbon and low-nitrogen medium, R2A, was an attempt to address these problems—at least for water environments (Reasoner and Geldreich 1985).

CULTIVATION OF MICROORGANISMS ON SOLID AND LIQUID SUBSTRATES

The existence of microorganisms in broth was first recognized by Antoine van Leeuwenhoek in the seventeenth century (Leeuwenhoek 1684). However, microbial cultures remained ill defined, and culture techniques were not yet developed at that time to a point where they could be used to characterize the properties of microorganisms. Cultivation of pure cultures of microorganisms on solid surfaces was finally developed by Robert Koch in the last quarter of the nineteenth century (Koch 1881). Originally Koch described gelatine-based substrates; later, agar was recognized as a gelling agent with superior characteristics for handling. The method was used to isolate and identify colonies grown from single bacteria that were immobilized on a surface or within a solidified gel. This breakthrough in microbiological technique overcame the difficulties of broth cultivation, where in the absence of isolation techniques the presence of microbial contamination could be evaluated only qualitatively. Since that time, many different compositions of nutrient substrates have been described to modify the growth-promoting properties of the media, but the main principles of Robert Koch's technique have been successfully applied for more than 100 years.

The reason for the long-lasting success of cultivation methods is that a surprisingly modern concept is applied: signal amplification. A single cell is amplified by growth until it becomes visible with the naked eye. The amplification factor necessary to achieve this is typically about 10^6-fold, which is difficult to reach with any signal amplification technique even today. The price to be paid for such tremendous amplification is time. Growth from single cells to a visible colony needs at least 18 to 24 hours, even for fast-growing microorganisms.

The principles of amplification by cell growth apply for cultures in liquid as well as on solid substrates. Solid substrate cultures allow the isolation of bacterial cell clones which can be characterized and further propagated as single-cell-derived genetically homogeneous cultures. In liquid laboratory cultures, more homogeneous growth conditions can be generated than on agar substrates. Also, cell numbers can be more easily monitored in the growing culture—e.g., by turbidimetric measurements. Thus it is much easier to study growth kinetics in liquid cultures of microorganisms.

Today, cultivation methods on liquid and solid substrates are well-established standard techniques in the microbiological laboratory, supplemented by biochemical and biophysical techniques. It must be recognized, however, that cultivation of isolated organisms in a laboratory is a highly specialized experimental setting, different from natural growth influenced by the complex conditions of a biofilm—e.g., species interactions, which cannot be simulated in the laboratory.

Growth Curves

Growth of microorganisms as measured in cultures in liquid media is usually described with the classical growth curve for bacterial populations, as illustrated in Figure 3.1. This growth curve can be divided into four distinct growth phases: The *lag phase,* the *phase of exponential growth* (log phase), the *stationary phase,* and the *reduction phase* (death phase).

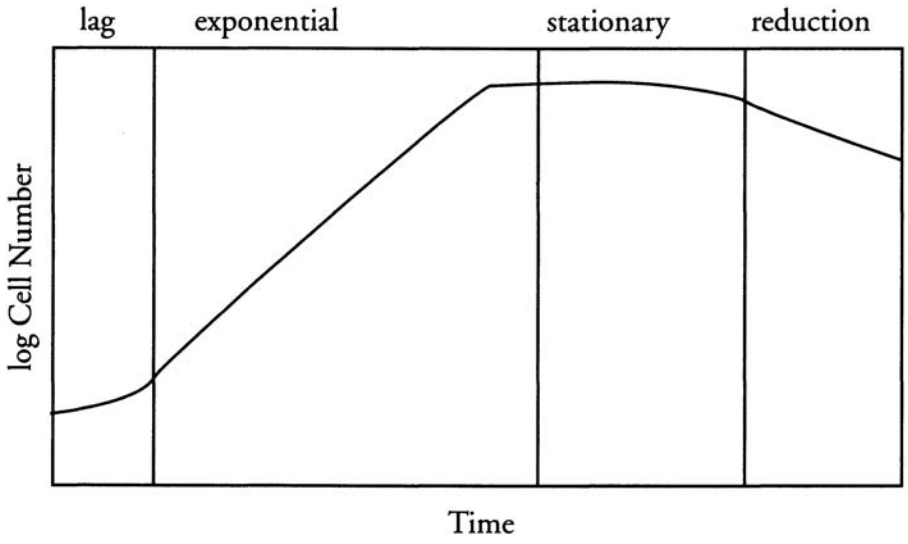
Lag Phase

When inoculated into fresh nutrient medium, a microbial population will usually need some time to adapt to the altered environment before growth begins. Depending on the physiological state of culture and the conditions offered by the nutrient medium, the lag time might be nonexistent or extended. An exponentially growing culture diluted into the same medium will continue growing without interruption (no lag phase). Sublethally damaged microorganisms (e.g., by dehydration or disinfection measures) may need a very extended recovery period. Duration of the lag phase generally depends on the nutrient medium used. It is well recognized, for example, that successful recovery of predamaged *Salmonella* depends on revitalization in a nonselective medium or buffer. Direct inoculation of samples into selective media will prolong the lag phase or even completely prevent recovery (International Standards Organization 1993).

Exponential Growth Phase

When the microorganisms are fully adapted to their new environment, growth in a homogeneous culture occurs by binary fission (cell division) at roughly constant time intervals. The time interval between two cell divisions is the *generation time* or *doubling time.* The length of the generation time is dependent on the species cultivated and on environmental conditions like the properties of the growth medium used, the incubation temperature, or the availability of oxygen (see Table 3.1). Duration of the phase of exponential growth and thus cell densities reached are also characteristic of the species under a given set of environmental conditions and can vary over a wide range. A fast-growing culture of microorganisms may, under optimal conditions reach overnight a stationary phase at about 10^9 CFU/mL, whereas a slow-growing culture under suboptimal conditions may continue growing for days and never reach a cell density of more than, e.g., 10^5 CFU/mL.

Figure 3.1 Growth curve for bacterial populations.



Stationary Phase

During exponential growth the environmental conditions in the culture are actively changed by metabolism of the culture. Essential nutrients are depleted from the medium, and excreted waste products from the metabolism are accumulated. The exponential growth is slowed down, and the culture finally reaches a stationary phase. The duration of the stationary phase depends on the organism and environmental conditions.

Table 3.1 Growth Requirements of Types of Microorganisms

Nutritional Requirements

- Autotrophic: Do not require organic source of carbon
- Heterotrophic: Do require organic source of carbon
- Phototrophic: Derive energy from photosynthesis
- Litotrophic: Derive energy from chemical reactions

Temperature Requirements

- Cryophilic: Optimum growth temperature about 1–20°C
- Mesophilic: Optimum growth temperature about 20–45°C
- Thermophilic: Optimum growth temperature about 45–115°C

Oxygen Requirements

- Aerobic: Oxygen required for growth
- Microaerophilic: Oxygen facultative for growth
- Anaerob: Oxygen inhibitory for growth

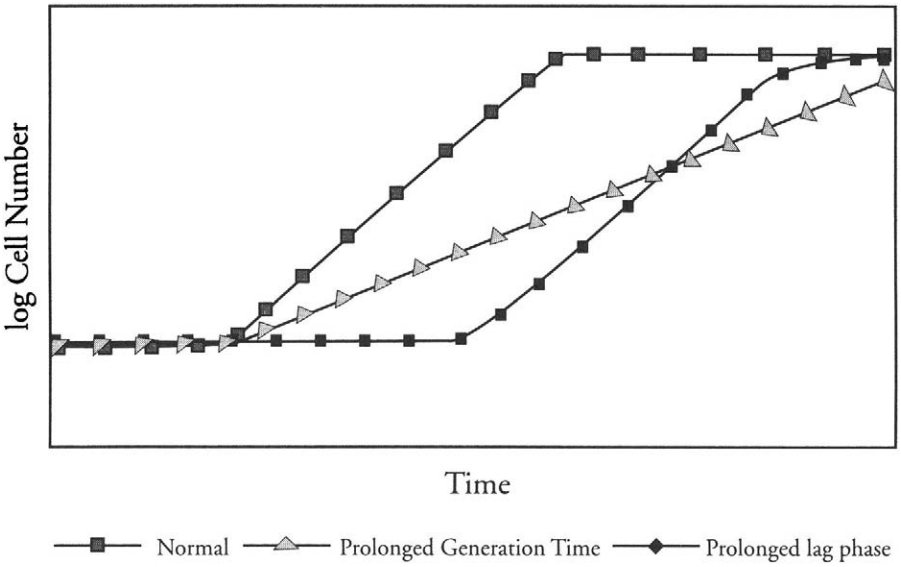
pH Requirements

- Acidophilic: optimum <6
- Neutralophilic: optimum 6-8
- Alkalophilic: optimum >8

Salt Tolerance

- Halophilic: >5% tolerated
- Moderately halophilic: 5–15% tolerated
- Extremely halophilic: >15% tolerated

Figure 3.2 Possible variations in the growth curve.



Reduction Phase

During the stationary phase, the microorganisms continue to metabolize, and thereby the environment is continuously changed. Typically the pH of the medium is changed, and toxic products accumulate. In most cultures of microorganisms, the number of viable cells starts to decrease after some time of stationary phase. Again this is dependent on the sensitivity of the cells and on the changes induced in the medium. A typical example of a toxic metabolic product is ethanol produced by *Saccharomyces cerevisiae,* which starts to kill the culture when an effective concentration is reached. Formation of bacterial endospores is induced during the end of the stationary phase and the beginning of the reduction phase.

Influence of Media Composition, Temperature, and Incubation Time

A very basic nutrient medium would consist of an aqueous solution of sugar as a source of energy, a buffered salt composition, and a vitamin mix to supply essential organic molecules. Most microorganisms, however, need a medium that contains a wider range of organic molecules. Digests of plant or animal proteins, yeast extract, or meat extract are typical additives that provide the complex mixture of organic molecules needed by most mesotrophic microorganisms. Such additives of natural origin cannot be easily standardized. Manufacturers of nutrient media select their tryptones and peptones to balance the growth-promoting properties of their media. Still, comparative studies on media show that growth of microorganisms is not identical in different media preparations of nominally identical composition. For this reason growth promotion tests for the media have been incorporated into the microbiological methods of the pharmacopoeias. It is interesting to note that in such studies identical inocula behave characteristically dependent on the properties of the medium; growth at different growth rates or delayed growth with identical growth rates can be observed (see Figure 3.2).

It is clear that no single medium or single set of conditions can accommodate all species of microorganisms. In “universal” media that support growth of a wide variety of microorganisms, pH is in the neutral range, the molarity of salt is in the physiological range, and a wide variety of organic molecules is available. Such media do not contain inhibitory substances. While some fastidious microorganisms will grow only on a blood-supplemented medium (e.g., *Neisseria* sp.), some microorganisms occurring in water will grow poorly when the medium is too rich. Other factors influencing the growth of microorganisms are compiled in Table 3.1, where microorganisms are grouped according to their growth characteristics under certain conditions.

Selective Media

Media designed to select certain microorganisms may contain a component or condition that is especially favorable for the species wanted. It is important to note, however, that most selective media contain substances that are inhibitory to microorganisms except for the species to be selected (and eventually some other species of similar growth characteristics). Such selective media may not offer especially favorable growth conditions; they just suppress any concomitant flora that could mask growth of the species to be selected and thereby allow their isolation.

Some selective media are even inhibitory to the selected species, but less so than to other species they are designed to suppress. A prominent example is some of the selective media for *Salmonella.* In such cases it is important to first revitalize any potential isolates that might not be fully active, by pre-cultivation in a nonselective enrichment step.

Growth media used in the microbiological tests of the European, Japanese, and U.S. pharmacopoeias and their purpose, selective principles, and indicator reactions are compiled in Table 3.2.

Indicator Reactions

In addition to nutrients and selective principles, specific indicators are incorporated into many growth media. Such indicators have the purpose of facilitating identification of microorganisms by indicating, usually by color change, metabolic reactions that only some organisms are capable of performing. Frequently, nutrients contained in the medium are metabolized to acids, which cause color change in chemical indicators like phenol red. Reduction of metallic ions to the metal may give colonies a glossy appearance; reduction of sulfates to sulfide causes blackening of the substrate around colonies. Changes in the turbidity of solid media by precipitation or dissolution of media components are also frequently used indicator reactions for the growth of microorganisms with specific metabolic capacities. By combination of several indicator reactions, some nutrient media are also strong identification tools—e.g., MacConkey agar, (MacConkey 1905) or xylose-lysine-deoxycholate (XLD) agar (Taylor 1965).

Other indicators may have the purpose of showing that critical characteristics are maintained and the medium is fit for use. The redox indicator used in thioglycolate broth is a good example.

Table 3.2 Nutrient Media of European, Japanese, and U.S. Pharmacopoeias

Medium	Source	Purpose	Elective or Selective Principle	Indicator Reactions	Reference
Casein soya bean digest broth or agar	E, J, U	Universal medium	None	None	
Lactose broth	E, J, U	Election of E. coli	None	None	
Sabouraud-glucose agar with antibiotics	E, J U	Selection of fungi, suppression of bacteria	High concentration of carbohydrates, low pH, antibiotics against bacteria	None	Sabouraud (1910)
Potato dextrose agar (Japan: with antibiotics)	J, U	Selection of fungi, suppression of bacteria	Potato extract and high concentrationof carbohydrates, low pH, antibiotics against bacteria	None	Beever and Bollard (1970)
Glucose-peptone agar with antibiotics	J	Selection of fungi, suppression of bacteria	High concentration of carbohydrates, low pH, antibiotics against bacteria	None	
Enterobacteria enrichment broth (Mossel)	E	Selection of enterobacteria	Inhibition of concomitant flora by brilliant green and ox bile	None	Mossel et al. (1963)
Crystal violet, neutral red, bile agar with glucose	E	Selection and indication of enterobacteria, suppression of concomitant flora	Inhibition of concomitant flora by crystal violet and bile acids	pH-dependent color change of neutral red, precipitation of bile acids	Mossel et al. (1962)
MacConkey agar	E, J, U	Selection of enterobacteria, suppression of concomitant flora	High lactose concentration, inhibition of concomitant flora by crystal violet and bile acids	pH-dependent color change of neutral red, indicating lactose metabolism, precipitation of bile acids	MacConkey (1905)
MacConkey broth	E	Selection of E. coli	Inhibition of concomitant flora by bile acids	pH-dependent color change of bromocresol purple, indicating lactose metabolism	MacConkey (1905)

Medium	Source	Purpose	Elective or Selective Principle	Indicator Reactions	Reference
Eosin-methylene blue (EMB) agar	J, U	Selection of enterobacteria, suppression of concomitant flora	High lactose concentration, inhibition of gram positives by methylene blue and eosin	Color reactions indieating lactose metabolism	Holt-Harris and Teagu (1916)
Tetrathionate broth	J, U	Selection of *Salmonella*	Inhibition of concomitant flora by tetrathionate and bile acids	None	Palumbo and Alford (1969)

Table 3.2 Nutrient Media of European, Japanese, and U.S. Pharmacopoeias (continued)

Medium	Source	Purpose	Elective or Selective Principle	Indicator Reactions	Reference
Tetrathionate bile brilliant green broth	E	Selection of *Salmonella*	Inhibition of concomitant flora by tetrathionate, brilliant green and bile	None	
Fluid selenite-cystine medium	J, U	Selection of *Salmonella*	Inhibition of concomitant flora by selenite	None	
Fluid Rappaport medium	J, U	Selection of *Salmonella*	Inhibition of concomitant enterobacterial flora by magnesium-chloride and malachite green	None	Rappaport and Konforti (1959)
Deoxycholate citrate agar	E	Selection and identifiedtion of *Salmonella*	Suppression of concomitant flora by high concentration of deoxycholate and citrate	pH-dependent color change of neutral red, precipitation of deoxycholate, thiosulfate reduction to black Fe-sulfide	Leifson (1935)
Xylose, lysine, deoxycholate agar	E, J, U	Identification of *Salmonella*	Some inhibition of concomitant flora by deoxycholate	pH-dependent color change of phenol red (indicating several possible reactions), thiosulfate reduction to black Fe-sulfide	Taylor (1965)
Brilliant green agar (BPLS-Agar)	J, U	Isolation and identification of *Salmonella*	Inhibition of concomitant flora by high concentration of brilliant green	pH-dependent color change of phenol red (indicating metabolism of lactose and saccharose)	Kauffmann (1935)
Bismuth sulfite agar	J, U	Isolation and identification of *Salmonella*	Inhibition of concomitant flora by brilliant green and bismuth	Bismuth-sulfide reduction to black Fe-sulfide; reduction of bismuth ions to metallic bismuth	Wilson and Blair (1927)
Triple sugar iron agar	E, J, U	Identification of *Salmonella*	None	pH-dependent color change of phenol red (indicating several possible reactions); thiosulfate reduction to black Fe-sulfide	Hajna (1945)

Table 3.2 Nutrient Media of European, Japanese, and U.S. Pharmacopoeias *(continued)*

Medium	Source	Purpose	Elective or Selective Principle	Indicator Reactions	Reference
Cetrimide agar	E, J, U	Isolation and differentiation of *Pseudomonas aeruginosa*	Inhibition of concomitant flora by cetylmethyl-ammoniumbromide (cetrimide)	None	Lowbury and Collins (1955)
NAC agar	J	Isolation and differentiation of *Pseudomonas aeruginosa*	Inhibition of concomitant flora by cetrimide and naladixic acid	None	
Pseudomonas agar for detection of fluorescein	J, U	Differentiation of *Pseudomonas* species	None	Stimulation of fluorescein formation	King et al. (1954)
Pseudomonas agar for detection of pyocyanin	J, U	Differentiation of *Pseudomonas* species	None	Stimulation of pyocyanin	King et al. (1954)
Baird-Parker agar	E, J, U	Isolation and differentiation of *Staphylococcus* species	Election of staphylococci by pyruvate and glycine; inhibition of concomitant flora by lithium chloride and tellurite	Clearance of turbidity, indicating lipase and protease action; reduction of tellurite to tellurium (black)	Baird-Parker (1962)
Vogel-johnson agar	J ,U	Isolation and differentiation of *Staphylococcus* species	Inhibition of concomitant flora by lithium chloride, tellurite, and a high concentration of glycine	pH-dependent color change of phenol red, indicating mannitol metabolism; reduction of tellurite to tellurium (black)	Vogel and Johnson (1960)
Mannitol-salt agar	J, U	Isolation and differentiation of *Staphylococcus* species	Inhibition of concomitant not halophilic) flora by high concentration of NaCI	pH-dependent color change of phenol red, indicating mannitol metabolism	Chapman (1945)
Reinforced medium for *Clostridia*	E	Election of *Clostridia*	Anaerobic incubation selects *Clostridia*	None	
Lactose sulfite medium	E	Election of *Clostridia*			
Columbia agar	E	Isolation of *Clostridia*	Inhibition of concomitant flora by gentamycin and anaerobic incubation	None	

Note: E=European, J=Japanese, U=United States.

Indicators for nutrient media continue to be developed. For example, fluorescent markers have recently been introduced that contribute to the sensitivity and specificity of the reactions. It is therefore helpful to occasionally revise the classical methods so that the methods of the compendia are adapted to the actual state of scientific knowledge.

Influence of Antagonistic Agents on Recovery

The presence of antagonistic agents in water, air, or on surfaces can also affect the recoverability of viable bacteria and fungi from pharmaceutical environments. For example, organisms damaged by chlorine, heat, or sublethal UV doses may not grow on solid or liquid media. Although the mechanisms responsible for this phenomenon are not well understood, direct viable-count techniques have shown that such organisms can actively respire following chemical or physical insult (Colwell 1993; Yu et al. 1993). Surface disinfectants, ozonated environments, water treatment compounds, and surface UV treatments may all induce this so-called viable, nonculturable state. These organisms are capable of growth, with the associated sequelae of product contamination by whole cells and by endotoxins (LPS).

Objectionable Microorganisms

The U.S. Pharmacopeia (1995) not only stipulates the maximum action limits for purified water and the microbial limits for raw materials, but also specifies that objectionable organisms may not be present. Similarly, current good manufacturing practices (cGMPs) militate for the absence of these organisms from manufacturing environments. Included in this group are coliform bacteria, which by compendial requirement cannot exceed limits promulgated by the U.S. Environmental Protection Agency (EPA). Currently a limit of <1 CFU/100 mL total coliforms is in place for water. Other compendially proscribed organisms include *Staphylococcus aureus, Salmonella typhimurium,* and some molds. The significance of organisms in nonsterile drug forms requires an evaluation with respect to the intended use, the nature of the preparation, and the potential hazard to the user.

Increasingly, however, noncoliform, heterotrophic bacteria previously considered as "normal inhabitants" of purified waters, raw materials, clean rooms, and other pharmaceutical environments are considered objectionable (Schulze-Robbecke et al. 1995). The prevailing view among most manufacturers and the U.S. Food and Drug Administration (FDA) is that objectionable organisms (other than coliform bacteria, which are prohibited entirely) are product application— defined. For example, purified water used in the manufacture of nonsterile ophthalmic preparations should be free of pseudomonads—in particular, *Burkholderia cepacia* and *Pseudomonas aeruginosa.* The definition of "objectionable organisms" in pharmaceutical manufacturing is likely to broaden in the future as new products and product applications are introduced. It follows that existing (including viable but nonculturable contaminants) and emerging environmental bacteria will be proscribed. Therefore, new detection systems must have the capability to recover, enumerate, and characterize new groups of organisms that may not have previously been characterized.

Finally, with the increasing recognition of the role bacterial biofilms play in the generation of bioburden in water systems, it is likely that limits will eventually be placed on both "normal inhabitants" and "objectionable organisms" associated with surfaces. Current limitations asso ciated with sampling and enumeration techniques for this significant subpopulation of water systems bioburden prevent the establishment of rational concern and action limits. Biofilms have been implicated as a reservoir for pathogenic bacteria in drinking-water distribution conduits (Wireman et al. 1993; Rogers et al. 1994), hospital water systems (Alary and Joly 1992), hemodialysis water systems (Phillips et al. 1994), and dental treatment units (Vincent et al. 1989; Pedersen 1990; Alary and Joly 1992; Schulze-Robbecke et al. 1995; Shearer 1996; Rohr et al. 1998). However, as McFeters et al. (1993) noted, no comprehensive studies on the microbial ecology of pharmaceutical-grade water systems—biofilm communities, in particular—have been carried out.

Noncultivable Microorganisms

As stated before, no single set of conditions exists that will accommodate all microorganisms. There is always a subset of species that cannot be detected by a given method. Some microorganisms may thrive only under conditions that cannot (yet) be simulated in the laboratory (e.g., microorganisms adapted to symbiosis in a biofilm). Microbiological limits can therefore be given only in the context of a given microbiological method. A different method will usually detect a different subset of the total microorganisms present in a material or location. Because the relative number of the various species of microorganisms in a population is not necessarily constant, different results may be obtained for the number of microorganisms detectable with different methods. When two methods are compared, method 1 may be superior to method 2 for cultivation of microorganisms from environment A, but inferior for cultivation of microorganisms from environment B or C, where different conditions cause the prevalence of other species. This must be considered if equivalence of alternative methods is to be demonstrated.

MICROBIOLOGICAL STANDARD METHODS OF THE PHARMACOPOEIA

Homogeneity of the samples is a problem of any microbiological test. In liquid samples, microorganisms may settle as sediment, remain at the surface (e.g., pellicle formation), or attach to the walls of a container (e.g., biofilm formation). In solid samples, infection may be localized and spread around the point of infection by local growth (e.g., mycelia of fungi). It is, therefore, important to take large enough representative samples and to carefully homogenize the sample.

There is also no assurance that every unit of a product to be tested will contain the same degree of microbial contamination. The edge of contamination that a unit of product may have received is highly unpredictable, depending on handling conditions. Storage conditions (e.g., for goods shipped in bags) may vary, allowing proliferation of microorganisms in some containers, but not in others. If the lot of a product to be tested consists of multiple units, a valid sampling plan is mandatory. Mixing of samples to a single composite sample is not generally recommended, because this may mask the presence of a single highly contaminated unit. Microbiological sampling plans should consider the sensitivity of the product toward

microbial contamination as well as the risk for the pharmaceutical process should microbial contamination of a container or lot be missed in testing.

For products that do not support the growth of microorganisms and are intended for use in less sensitive final products like solid oral dosage forms, supervision of the general microbiological status by skip-lot testing is common practice. For microbiologically sensitive products, lot by lot testing should be mandatory.

Neutralization of Antimicrobial Activity and Validation of Recovery

Another important consideration for microbial testing is the possible presence of antimicrobial activity in the sample. Products may be preserved or have inherent antimicrobial activity. The environment encountered in pharmaceutical microbiological testing is also subject to frequent sanitation measures, so residues of disinfectants may be present in the samples. Inactivation of antimicrobial activity is important to allow reliable recovery of contaminating microorganisms even if sublethally injured.

The pharmacopoeial methods ask for validation of the test, by inoculating test samples with a given set of microorganisms as listed in Table 3.3. This validation method does not, however, account for the presence of sublethally injured microorganisms that may recover in the product after it has been tested, and lead to spoilage or present a danger to the patient. Nor does it account for the recovery of microorganisms that are less adapted to the conditions of the test as fast-growing laboratory strains.

Total Viable Count

This test is internationally accepted with very few modifications. Casein peptone—soybean digest medium—contains a mixture of animal- and plant-derived organic molecules. The range of microorganisms detected is quite broad. It includes gram-negative rods, gram-positive cocci, bacilli, and other gram-positive rods. Facultatively anaerobic microorganisms like Corynebacteria, which populate the skin of personnel, are rarely detected.

The test will miss highly fastidious microorganisms that require serum or blood additives in the medium. The test method is also not well suited to isolate microorganisms adapted to low-nutrient environments, like pure water systems. For this reason it has recently been recommended to use different media for water testing (U.S.Pharmacopeia 1995; European Pharmacopoeia 2002). It is of major importance that the strategy of microbiological testing and the limits set be chosen under due consideration of further processing, downstream handling, or storage of the product tested. Compendial methods can only give a minimumtesting requirement and should take the purpose of the test into consideration. To characterize and validate a water system, different media are necessary and may give valuable additional information. To characterize water as a pharmaceutical excipient, it is important to understand the relevance of any additional recovered microorganisms for the pharmaceutical process. If the water is to be sterilized by filtration and thereafter aseptically processed, the numbers of bacteria detectable by any method will be highly important. If the water is used for terminally sterilized parenteral dosage forms, the absence of endotoxins may be of much greater significance than any number of aerobic microorganisms detectable by a standard or nonstandard culture method.

Table 3.3 Microorganisms for Validation of Microbiological Methods as Listed in the European and U.S. Pharmacopoeias

Test Organism	Test Strain	Purpose
Bacillus subtilis	ATCC 6633, CIP 52.62, NCIB 8054	(E) Sterility 1 (U) Sterility 3 (E) TVC 1 (E) TSM
Clostridium sporogenes	ATCC 19404, CIP 79.3	(E) Sterility 2
Clostridium sporogenes	ATCC 11437	(U) Sterility 1, 4
Escherichia coli	ATCC 8739, NCIMB 8545, CIP 53.126	(E) TVC 1 (E) TSM
Pseudomonas aeruginosa	ATCC 9027, NCIMB 8626, CIP 82.118	(E) Sterility 1 (U) Sterility 1 (E) (U) EAP
Staphylococcus aureus	ATCC 6538 P, CIP 53.156, NCTC 7447 NCIB 8625	(E) Sterility 1 (E) TVC 1 (E) TSM
Staphylococcus aureus	ATCC 6538, NCTC 10788, NCIB 9518, CIP 4.83	(U) Sterility 1 (E) TVC 1

Test Organism	Test Strain	Purpose
		(E) TSM (E) (U) EAP
Candida albicans	ATCC 10231, IP 48.72, NCPF 3179	(E) Sterility 3 (U) Sterility 3 (E) TVC 2 (E) TSM (E) (U) EAP
Candida albicans	ATCC 2091, IPI 80.79	(E) Sterility 3 (E) TVC 2 (E) TSM
Aspergillus niger	ATCC 16404 IMI 149007, IP 1431.83	(E) Sterility 3 (U) Sterility 3 (E) (U) EAP

Test conditions and requirements for European (E) and U.S. (U) pharmacopoeias:
(E) Sterility 1: Ph.Eur. 1998: Sterility Test, Thioglycolate, Aerobic Growth: 32.5±2.5°C, 3 days.
(U) Sterility 1: USP 23 Suppl.8: Sterility Test, Thioglycolate, Aerobic Growth: 32.5±2.5°C, 7 days.
(E) Sterility 2: Ph. Eur. 1998: Sterility Test, Thioglycolate, Anaerobic Growth: 32.5±2.5°C, 3 days.
(E) Sterility 3: Ph. Eur. 1998: Sterility Test, Casein Soy Bean, Fungi, Aerobic Growth: 22.5±2.5°C, 5 days.
(U) Sterility 3 USP 23 Suppl. 8: Sterility Test, Casein Soy Bean, Fungi, Bacteria, Aerobic Growth: 22.5± 2.5°C, 5 days.
(U) Sterility 4: USP 23 Suppl.8: Sterility Test, Alternative Thioglycolate, Aerobic/Anaerobic Growth: 32.5± 2.5°C, 7 days.
(E) TVC 1: Ph.Eur. 1997: Microbial Contamination Total Viable Count, Bacteria, Casein Soy Bean, GROWTH: 32.5±2.5°C, 5 days.
(E) TVC 2: Ph.Eur. 1997: Microbial Contamination Total Viable Count, Fungi, Sabouraud Dextrose with Antibiotics, 22.5±2.5°C, 5 days.
(E) TSM: Ph. Eur. 1997: Microbial Contamination Test for Specified Microorganisms, Recovered in Test.
(E) (U) EAP: Ph. Eur. 1997; USP 23 Suppl. 8: Efficacy of Antimicrobial Preservation. Decrease of viability according to test criteria.

Yeast and Mold Count

Pharmacopoeial yeast and mold counts are performed on highly selective media. Sabouraud agar is selective by its acidic formulation, growth at reduced temperature, and addition of a combination of antibacterial antibiotics. Such conditions are necessary to suppress overgrowth of bacteria from a highly contaminated test sample. While this may be necessary in clinical (e.g., stool) samples, there is no need to use stringent selectivity in pharmaceutical samples where microbiological contamination is low. In such samples (with the exception of herbal remedies) it is much more important to avoid masking of the bacterial flora by overgrowth of mold mycelia than the other way around. Yeast and mold in most instances grow as well in neutral nonselective media and 30 to 35°C as on selective media at 20 to 25°C. The only obvious reason to use selective media is to obtain a separate specific count for yeast and mold, which may be important for investigation of the origin of a given contamination.

Molds are typically growing in mycelia and cannot be easily quantitated by counting colonyforming units, unless reference spore preparations are used as test suspensions. Thus, the significance of mold counts by the pharmacopoeial test method is questionable.

Specific Microorganisms

The pharmacopoeias traditionally require absence of specified microorganisms (see Table 3.4) from excipients, active ingredients, intermediates, and finished dosage forms. This concept is being questioned today. The list of specified organisms certainly does not comprise all objectionable microorganisms; such a list would include all relevant pathogenic organisms as well as organisms able to proliferate in a given product or environment. A complete list of all possible objectionable microorganisms would be very extensive; to conduct specific tests to exclude all these organisms would be costly and impracticable.

The significance of the organisms as indicators for a certain route or type of contamination is also questionable. Enterobacteria may be seen as indicators for contamination from a moist environment. Indication of fecal contamination can be assumed for coliform microorganisms (as traditionally tested for in water microbiology). The general role of *E. coli* as an indicator for fecal contamination is quite well established, and at least some strains are dangerous pathogens. *Salmonella* is clearly an objectionable genus because of the pathogenicity of all the member species, but a question needs to be raised about why other pathogenic members of the enterobacteria like *Shigella* sp. are considered less objectionable. The pathogenic species *Pseudomonas*

Table 3.4 Significance of Specified Microorganisms

Microorganism	Significance
Enterobacteriaceae	General indicator for wet contamination General indicator for hygienic conditions
Escherichia coli	Indicator for fecal contamination Pathogen
Salmonella sp.	Pathogen
Staphylococcus aureus	Indicator for skin contamination Pathogen
Pseudomonas aeruginosa	Indicator for contaminated water Pathogen
Clostridia	Indicator for anaerobic contamination

aeruginosa is also considered an indicator of contamination from an aquatic environment, but its relevance in this context is questionable. Organisms like *Burkholderia (Pseudomonas) cepacia* would be more appropriate for this purpose, and they are missed by the selection on cetrimide agar. *Staphylococcus aureus* is unquestionably pathogenic, but a better indicator organism for contamination from human interference would certainly be a more frequently isolated one like *Staphylococcus epidermidis.*

Test for Sterility

This test is still considered a gold standard for release of sterile pharmaceutical products, even though it is very clear that the test is nearly meaningless for batch release from a statistical point of view (Spicher and Peters 1975). At least for products sterilized in their final container, the samples tested are more prone to inadvertent contamination than the product coming from the production process

In addition, the testing method is far from perfect. Even though growth media and conditions are designed to detect a wide variety of organisms, there can be no simple set of conditions that could detect all organisms present. During method validation it must be demonstrated that test organisms can be effectively cultivated under simulated test conditions. It is very difficult, however, to make sure that this would also apply to any nonadapted and sublethally injured microorganisms possibly present in the product. Although laboratory-grown microorganisms are expected to grow to visible turbidity within 2 to 3 days, positive results in actual testing may become detectable only after 7 to 10 days or more. It would have to be established for each case whether this late appearance of turbidity represents a prolonged lag phase or a very long generation time during exponential growth (see Figure 3.2). This difference may be decisive for rapid methods involving early detection of growth.

With all the limitations of microbiological testing, there can be no assurance that the test as described in the pharmacopoeias will detect every single contaminant in a product, but every alternative method must be measured against this theoretical limit of detection. An alternative method relying on a different set of conditions might detect a different subset of all possible organisms. Under such circumstances, equivalence of the methods would be very difficult to define. Typical positive rates for sterility tests performed in conventional clean rooms are at about 0.1% (Doorne et al. 1998), and the number of tests done per year in a typical sterility testing laboratory is between 500 and 5,000. Several years of parallel testing (including the risk of introduction of false positives in each of the test series) would be needed to obtain statistically significant figures demonstrating method equivalence.

The advantage of the compendial cultivation method used in the test for sterility is the availability of any isolate for identification and other characterization. Because characterization is of prime importance for the failure investigation to be carried out by the pharmaceutical manufacturer, capability to retrieve the detected organism would be a prerequisite for the acceptance of any alternative method.

Antimicrobial Effectiveness Tests

The cultivation methods used in the antimicrobial effectiveness test are identical to the total viable count, the purpose of the test is to recover test organisms that have been inoculated into a given product. The critical problem is inactivation of the antimicrobial agent to allow recovery of the surviving inoculum organisms after the test period. Methods that do not require growth of the organisms but rely on direct recognition of viable cells may be easier to apply and give as meaningful results as the standard cultivation test.

Summary and Outlook

Although the methods described in the pharmacopoeias are the officially recognized ones in the pharmaceutical field, revision is regularly needed to verify that the methods still represent the current state of technology. Recent technological developments in the fields of food and water microbiology may be better suited for the purpose of pharmaceutical microbiology than some of the methods that were originally described for the purpose of medicinal microbiology.

Alternative methods that lend themselves to automation and give faster results than the classical cultivation methods are now becoming available. To use these attractive new concepts, pharmaceutical manufacturers have to make sure that these alternative methods are equivalent to the classical methods. With the strength but also the limitations and ambiguities of the cultivation methods, "equivalence" may be very difficult to define. It will be important to understand and accept that new methods as well as the classic methods have strengths and shortcomings that will have to be weighed against each other.

Care must also be taken to avoid a push toward requirements just because microorganisms that have been uncultivable with the classical methods can be detected with alternative methods. This would inevitably lead to a further increase in the technical standards required for the production of pharmaceuticals. Given ever-increasing healthcare costs, additional technical requirements can be justified only if a real threat to patients has to be minimized. The significance of such findings must be carefully evaluated, of course. At present, there is no indication that microbiological populations unrecognized by the classical methods cause a significant health hazard in the production of pharmaceuticals.

Within the present effort for international harmonization, the pharmaceutical methodology for specific microorganisms should be reconsidered, to design a modern and practicable concept to assure that microbiological manufacturers can comply with the requirement that their products be free from objectionable microorganisms, with a reasonable margin of safety.

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4
Overview of Alternative Rapid Microbiological Technologies

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Several factors affect the adoption of faster, cheaper, and more sensitive microbiological techniques in the pharmaceutical industry. The following is an overview of industry needs and requirements, drivers for change, and examples of available technologies. These techniques may have an impact on the quality assurance of pharmaceutical products, whether in new product development or during the manufacture and approval of existing formulations.

Laboratory efficiencies are not always improved by the introduction of expensive capital equipment, however. Time taken to understand the types and volumes of tests performed and other associated laboratory practices can lead to the selection of alternative methodologies that maximizes the current microbiological testing.

INDUSTRIAL NEEDS AND REQUIREMENTS

The pharmaceutical industry today faces a period of unprecedented change. Pressure is coming from worldwide restructuring of healthcare systems, with increased demand for cheaper therapies and greater cost effectiveness. Development and manufacturing costs are rising at the same time as profits are being squeezed. This situation inevitably leads to increased pressure on every aspect of the drug development and manufacture process. The future requirements for drug development and manufacture are clear:

- Costs must be cut across the drug development process.
- Lead times in development and manufacture must be reduced.
- No compromise in product quality can be allowed.

Microbiological test methods based on nineteenth century techniques are increasingly failing to meet the demands being placed on today's pharmaceutical industry. Faster, cheaper, and more sensitive test methods are required. There is no value in producing products quickly and efficiently only to find that they are locked up in warehouses for weeks or months waiting for microbiological test results. "Just in time" manufacture is the way of the future; microbiological test methods must change to meet this challenge.

Traditional microbiological test methods have relied on the growth of microorganisms. Results take days or, as in the case of the sterility test, weeks. Microorganisms grow too slowly to give rapid results. A fundamental shift in test methodology is therefore required. Interesting pointers to the future are coming into focus. New test methods will rely on the detection of cellular components and biochemical and genetic markers that will not require cell replication. Amplification of such things as microbial adenosine triphosphate (ATP), nucleic acids, and enzyme activity will produce results in minutes or hours instead of days (Lundin 1989, Stager and Davis 1992, Wills et al. 1997a).

Why Test?

Microbiological testing of sterile and nonsterile product formulations is essential for several reasons:

- patient safety;
- product quality;
- regulatory and legal requirements; and

- increased understanding of processes.

Safeguarding patient safety must be the primary objective of any manufacturing analytical technique. Failure to safeguard can have devastating effects on both patients and offending companies. The very nature of some diseases can create huge demands for rapid product manufacture and release. This situation is particularly true during epidemics. Quicker product release with faster, more sensitive test methods would be a huge benefit to patients and manufacturers during such extreme circumstances. The time required for results from traditional microbiological test methods such as the sterility test are getting longer, not shorter. The recommended incubation period has been increased to 14 days, compared with 7 days previously. Nonsterile product testing as recommended by the U.S. Pharmacopeia is 5 days. Clearly such test periods will put pressure on product release. Faster product release would help meet patient demand and reduce manufacturing costs, thus helping to lower therapy costs overall.

Product quality is ensured through good manufacturing practice (GMP). A fully validated and effectively controlled manufacturing process is vital for product safety and quality. Microbiological monitoring of the process is an essential way of demonstrating process control. Once validated, critical process parameters must not alter significantly. If they do, process control is lost and product quality and/or safety may be compromised. Effective control of the manufacturing process is demonstrated by microbiological in-process monitoring of product, environment, and operators. Real-time results would significantly help toward process control. Faster results would allow more responsive corrective action. These factors would aid in reducing batch rejection and lost production, thus helping to keep manufacturing costs down. Traditional monitoring methods take several days. As manufacturing processes become more efficient, more batches will be produced during the time taken to get microbiological results. When process control is lost, the number of batch rejections increases. Fortunately such loss of control is a rare event. However, when a single batch of pharmaceutical product can cost hundreds of thousands of pounds, the potential for significant financial losses is very real. Rapid microbiological methods will help reduce the possibility of batch rejection caused by compromised manufacturing conditions.

The pharmaceutical industry is one of the most heavily regulated industries in the world. Microbiological test methods must conform to tight regulations. Methods are developed and validated in compliance with international pharmacopoeias such as the U.S. Pharmacopeia and the European Pharmacopoeia. Conventional microbiological methods have changed little over the course of the twentieth century. As a consequence regulators are accustomed to seeing a limited number of microbiological assays. These methods are widely used and are accepted by the various regulatory authorities. Add to this the extremely conservative nature of pharmaceutical microbiology and the cost of drug development, and you have a very unfavorable situation for the introduction of new techniques.

RAPID METHODS AND THE DRUG DEVELOPMENT PROCESS

The drug development process can be divided into three broad areas:

- Research,
- Development, and
- Manufacture.

During the research phase, new chemical entities (NCEs) are produced from natural sources and chemical synthesis. These compounds are screened for signs of pharmaceutical activity before they go on to preliminary toxicological evaluation. In the past this screening process has been somewhat random. Huge banks of microbial isolates and natural products have been evaluated. This situation is changing rapidly. Automation, robotics, biotechnology, and combinatorial chemistry are having a huge impact on drug discovery. Combinatorial chemistry is slashing the time taken to develop NCEs. It is also expanding the potential number of candidate compounds because of the precise way in which active molecules can be manipulated. Biotechnology is now beginning to have an impact also. New biological entities (NBEs) are emerging. The message here is that the number of active compounds coming into drug development pipelines is increasing at an alarming rate. New rapid screening methods that will allow the efficient processing of large numbers of compounds are required. Current pharmacopeia methods such as preservative efficacy and antibiotic screening are too labor intensive. New methods that will allow high throughput evaluation of potential NCEs and NBEs are needed.

During the development phase, potential active compounds are taken into preclinical and clinical evaluation stages. A huge amount of information is generated on the pharmacology, toxicology, chemistry, and microbiology of the active compound and its route of manufacture. Synthesis, scale-up, and manufacture processes are developed, evaluated, and finalized. Drug stability programs are developed and are run for up to several years. Analytical methods are developed and validated. The development phase culminates in the production of a regulatory submission. A product is ready for sale only when a manufacturing license has been granted. This whole process takes several years. The active compound will be under patent by

then, so the longer the development process takes the shorter the patent life for the final product. Drug development is expensive, costing in the range of £200 M to £300 M. Rapid methods are needed in the development phase for both in-process testing and product release testing. Details of the product release test will have to be registered in the regulatory submission. The FDA and European regulatory authorities will need to scrutinize any new method. Details of the test and validation data will need to be supplied, along with a justification and demonstration of equivalence with more traditional methods. It is for this reason that dialogue among the regulatory authorities, manufacturers of new technologies, and potential users is essential. It is quite unthinkable that regulatory submission for a new product could be delayed due to the introduction of unfamiliar microbiological test methods. Years of work could be wasted and revenue lost. This situation begins to explain the innate conservatism of the pharmaceutical industry. It also underlines the difficulties experienced by unwitting technology suppliers accustomed to less regulated markets.

During the manufacturing process, the registered product is produced for sale to the public. Microbiological data are generated in three main areas: raw material testing; in-process controls, including environmental monitoring; and product release testing. The degree and amount of testing will depend on whether the product is sterile or nonsterile. Bottlenecks will be process dependent. Real-time analysis is not yet possible. Real-time data for raw materials and in-process testing would significantly contribute to reducing production losses. Emerging technologies offer the potential of single cell detection within 2 to 3 hours; examples are the ScanRDI and bioluminescence, particularly adenylate kinase.

Cost

A major consideration in the successful introduction of new technologies into industrial microbiology is cost. In general, increased sensitivity requires expensive detection systems. The use of such techniques will depend on the size and wealth of the manufacturing company and the relative value of the product involved. It is important to consider the overall savings a new method will create in the manufacturing process. The method may require specialist advice. Software to assist nonfinancial specialists to do this sort of complex economic evaluation is increasingly available. Process bottlenecks and estimated losses due to batch failure or recall must be considered. This type of economic evaluation is not normal practice for the pharmaceutical microbiologist. In fact it is not common practice at all to consider the impact of individual parts of a manufacturing process on the overall efficiency of the system. Such analysis is essential, however, if the real benefits of rapid methods to the overall process are to be realized. It is an encouraging sign that many pharmaceutical companies are beginning to consider process optimazation.

New Method Requirements

New methods have been introduced very successfully in the food and beverage sectors over the past 20 years. These technologies have been given the collective name of *Rapid Methods* and include ATP bioluminescence, impedance, the direct epifluorescent filter technique (DEFT), and others. To date none of these techniques have been widely used by the pharmaceutical sector, in part because of a lack of sensitivity of many of these rapid methods. However, a more fundamental problem has been the failure of the technology suppliers to understand the specific technical and regulatory requirements of the pharmaceutical sector. These requirements are very different from those of the food and beverage industries. Initially it was hoped that methods from one industry could be easily transferred into the pharmaceutical sector. There was little, if any, communication between the manufacturers of these techniques and the potential users in pharmaceutical microbiology. As a result little technology transfer was seen between the different industry sectors. The suppliers appeared to overpromise and underdeliver. This has created a considerable amount of skepticism among pharmaceutical microbiologists toward rapid methods in general.

Because the pharmaceutical sector is highly regulated and conservative, introduction of any new method is a complex issue. Successful introduction of new test methodology into pharmaceutical microbiology requires several ingredients:

- fitness of purpose (the ability to do the job);
- validation package and support;
- regulatory acceptance; and
- time, money, and commitment.

"Fitness of purpose," an expression gaining ground in the area of rapid methods, means that the test method in question must be able to do what it says it can do. The ability to do the job is a little more difficult to demonstrate, for the following reasons. Equivalence with a reference method is required. Unfortunately many of the "gold standard" reference methods, such as membrane filtration and agar plate methods, are not perfect. They can be less sensitive than emerging techniques, and direct comparison can result in poor correlation. A good example is microbial enumeration using ATP bioluminescence compared to

agar plate methods. In bioluminescence the amount of light produced by an enzymatic reaction, luciferin-luciferase, is used to quantify the number of microorganisms present. The amount of light produced is directly proportional to the ATP present. Pour plating, on the other hand, requires the growth of individual organisms on nutrient media to produce visible colonies. Microorganisms in nature, however, tend to exist in clusters, not as single organisms. The nutrient media and conditions used can adversely influence the recovery of microorganisms, particularly if they are stressed in the transition from natural environments. The direct comparison between microbial ATP content and the ability to grow on nutrient media can therefore lead to differences between bioluminescent-based methods and traditional plate techniques.

Validation is the process required to demonstrate that a given method can achieve what it sets out to do. With rapid methods validation is an important consideration. Software and hardware must be fully validated to the requirements of GMP. Documented proof must be made available to prospective users and regulatory bodies. It is also vital that the user and the supplier of new techniques design a suitable performance qualification (PQ). The PQ must be able to demonstrate accuracy, sensitivity, robustness, and suitability of the new method to the task at hand. Guidelines on how to implement and validate microbiological rapid methods are beginning to emerge. One such document is the Parenteral Drug Association Technical Report No. 33 "Evaluation, Validation, and Implementation of New Microbiological Testing Methods." This document is an invaluable guide to anyone interested in implementing microbiological rapid methods.

Above all, dialogue among the industry user, technology supplier, and regulatory authorities is essential. Encouraging signs that this process has started are that new techniques are emerging and regulatory approval is being given. In 1997 the UK's Medicines Control Agency approved the use of an ATP-based method for the rapid screening of nonsterile product testing (Weatherhead 2000). This new breed of rapid methods results from close collaboration of government agencies, the industry, and the rapid method manufacturers (Wills et al. 1997b). An important difference from earlier attempts is not the technologies themselves; they are in fact similar to earlier methods. Instead, the difference is the way in which these methods have been designed to satisfy the needs of the pharmaceutical sector in both functional and regulatory aspects.

AVAILABLE TECHNOLOGIES

Many technologies are available to facilitate sample preparation, media preparation, sample processing, result recording, and isolation of specific organisms (also see Chapters 5 and 8). These are areas largely ignored by the manufacturers of the expensive systems. However, using some of these techniques can free up resources, and significant efficiencies can result. An additional benefit is that enhancements to preparation methods are easier to validate and more readily acceptable to regulators.

Sample Preparation

The tedium of weighing a sample and applying the correct amount of diluent can be offset by the use of the gravimetric diluter (e.g., Diluflo™ by Spiral Biotech, United States). This equipment automatically dilutes solids, semisolids, and liquid samples in dilution increments of 0.1% and with a balance accuracy of 0.1 g (Manningen and Fung 1992). The system, in conjunction with filtration, can supply sterile diluent and can dispense presterilized media.

When a sample has been weighed and the appropriate diluent has been added, dispersion is usually performed by vortexing or blending in stainless steel blenders. In the food industry the Stomacher® (Seward Medical, United Kingdom) developed by Sharpe and Jackson (1972), has been widely accepted. There is no reason why this technology cannot be used within the pharmaceutical industry. The Stomacher allows for a sample to be dispersed in a sterile plastic bag by the pummeling action of paddles squashing the sample from side to side in the bag.

A new variant called the Pulsifier® (VWR Scientific Products, United States, Microgen Products, United Kingdom) does not crush the samples, but uses an oval metal ring surrounding the plastic bag to apply a high frequency beating action, combined with shock waves and stirring, to drive microbes into suspension (Fung et al. 1998). The Pulsifier generates fewer particles than the Stomacher and thus expands its compatibility to other enumeration and isolation systems, such as bioluminescence, flow cytometry, and the polymerase chain reaction (PCR).

Media Preparation

It is possible to use prepared media for a variety of applications—e.g., enumeration or isolation. Companies such as bioMérieux SA (France) and Difco Laboratories Ltd. (United Kingdom) supply a wide range of solid and liquid media. The media can be supplied in bulk or individual plate or tube formats for testing. General-purpose media (total viable counts, Staphylococci, coliforms, yeast, and molds), chromogenic substrates (e.g., *Candida albicans, Escherichia coli, Salmonella* spp.) and specific isolation agar (e.g., *Mycoplasma* spp., *Helicobacter* spp., *Neisseria* spp.) are available, as well as specialized systems for sterility testing and susceptibility testing. The benefit of prepared media comes from savings in time

and resources especially in media preparation and autoclaving. In addition quality assurance benefits related to traceability can be obtained. However, such media may be expensive, and a thorough review of usage and benefits versus costs needs to be undertaken. One application that is gaining in popularity is the use of pre-prepared media for environmental monitoring. Both Difco and bioMérieux supply triple-wrapped, irradiated media for settle plates. In-house preparation of environmental monitoring media is time consuming and may introduce extraneous contamination.

An alternative to media for isolation and enumeration of microorganisms is Petrifilm™ plates (3M Microbiology Products, United Kingdom), a system that comprises 2 dry, rehydratable films coated in nutrients, a cold-soluble gel, and tetrazolium indicator dye for colony enumeration. It is very simple to use: 1 mL of prepared sample is placed onto the lower, gridded, film; the upper film is allowed to cover the sample and the sample is spread evenly across a circular area on the lower part; the films are then incubated for 24 hours at the appropriate temperature.

The main advantage of Petrifilm plates are that they remove the need for media preparation, inoculation of films is easy, and savings in storage and incubator space are realized. One additional benefit for the pharmaceutical industry is that Petrifilm plates can be used for environmental monitoring. They can be used for air sampling (settle plates), for contact surface sampling, and also in conjunction with swabs.

Benefits may be gained from the use of on-demand smaller volumes of media. The MikroClave™ (Spiral Biotech) uses microwave technology to sterilize media: e.g., 100 petri plates in 10 minutes, 10 plates in 45 seconds. Other labor-saving systems are available for streak plates (Isoplater 180, Vista Technology Inc., Canada), spiral plates (Autoplate® 4000, Spiral Biotech), and colony counting using CASBA™ 4, image analyzer and CASBA™ 2 laser colony counting systems from Spiral Biotech. Several image analyzers are available.

Membrane Filtration

Within the pharmaceutical industry, the method of choice for microbiological analyses is membrane filtration. However, all microbiologists who use membrane filtration are familiar with the care that needs to be taken in handling the filters. As a consequence, several membrane-based devices for microbiological analyses have been developed by various companies. The sophistication of the devices varies depending upon their application. For example, the Microfil™ system (Millipore Corp., United States) uses pre-packed 0.45 µm membranes with a patented vacuum support and disposable funnel. Microfil™ reduces manipulations and the extent of equipment sterilization, while the membrane lifting device enhances efficient membrane transfer. An extension to Microfil is the Milliflex™ 100 system, in which the disposable funnel and membrane are combined as a single pre-sterilized unit. The combined unit is placed on the Millipore vacuum system, the sample is filtered and the filter assembly then snapped into a prefilled medium cassette, and the membrane is then incubated.

Water system (purified water for injection) sampling is a critical process requiring trained samplers. The Milliflex P system minimizes extraneous contamination. This closed system comprises a 0.45 µm filter membrane within a cassette, which can be directly attached to a sanitary design sampling port, and water samples are filtered directly. After the sampling, the filtration unit is directly coupled to a prefilled media cassette and incubated

The Steritest™ system from Millipore is a membrane-based device that is attaining significant use in the pharmaceutical industry. Steritest is a closed method for sterility testing, designed to be compatible with a range of dosage forms and packaging types—e.g., bottles, vials, ampoules, syringes, collapsible bags, antibiotics, creams and ointments, aerosols, and liquids. In practice the Steritest is used in conjunction with prepared media, thereby reducing the need for laboratory resources in the production and maintenance of media and diluents. The Steritest reduces the risk of false positives by minimizing technician manipulations and guarding against adventitious contamination. However, it still requires 14 of days incubation.

An addition to this system is the Steritest Integral 316 II designed to fit into the work surface of laminar flow hoods or isolators, thereby allowing only components necessary for the test to be exposed within the "clean environment." Millipore offers training and validation support for the Steritest system.

Specific Organism Isolation

Defined substrate technology involves the linking of an enzyme substrate to a colorless chemical indicator such that when the substrate is used a change in color or fluorescence is observed. The most familiar systems are the media developed by incorporating O-nitrophenyl-B-D-galactopyronoside (ONPG) to detect coliforms and 4-methyl-umbelliferone glucuronide (MUG) to detect *E. Coli* (using ultraviolet light). Commercially available media have been developed—e.g., Colilert™ and Coliquick™ (IDEXX Labortories, United States), and Colifast® (Colifast AS, Norway)—based upon ONPG and MUG. A range of alternative agars incorporating different dyes, substrates, and selective agents are available—e.g., BBL CHROMagar™ (Becton Dickenson, France), Chromocult™ (Merck, Germany), BCM™ agars (Biosynth International Inc., United States), M-Coli blue (Camlab, United Kingdom), Rainbow® agars (Biolog, Inc., United States), and Simplate (IDEXX).

The advantage of such agars lies in the increased speed of characterizing the presence of specific organisms without laborious resuscitation and enrichment stages. However, the validation of the media must be undertaken carefully, because the enzymes used as the basis of detection are found in many genera; thus, the combination of specificity and selectivity must be assured. Although the media costs appear to be high initially, when one considers the time saved and the reduction in the number of different media required to obtain the same result, defined substrate technology may be cost-effective.

Isolation and separation of specific microorganisms by use of selective media and enrichment broths may be enhanced by the use of immuno-magnetic separation (IMS). IMS is a non-growth-related procedure that utilizes super-paramagnetic beads, linked to antibodies or lectins, that bind to specific organisms. Super-paramagnetic beads are supplied by Dynal (United Kingdom), Vicam (United States) and Metachem Diagnostics Ltd. (United Kingdom).

These beads will attach to target organisms, and after the specified incubation period (10–60 minutes) an external magnet is applied to the suspension, and the beads are segregated from the rest of the sample matrix.

IMS can be linked to other rapid microbiological technologies, such as impedance, immunoassays, and DNA hybridization and amplification technologies. Isolating specific organ isms from competing flora and any inhibiting substances should reduce the time to detection. It must be remembered that although IMS will isolate stressed organisms, it will also recover dead organisms that may cause false positives in polymerase chain reaction (PCR) systems.

Anaerobes

Pharmaceutical companies perform tests for anaerobic organisms to varying degrees, from non-routine environmental monitoring sampling to analyses on every finished product batch. Consequently the level of sophistication in terms of equipment varies to meet the need. Oxyrase® (Oxyrase, United States) is a membrane fraction of *E. coli* that can convert oxygen to water, thereby reducing oxygen tension and creating anaerobic conditions. Such anaerobic conditions can be maintained in both liquid and solid media. When used in conjunction with the Oxydish™ (Oxyrase, Inc.), an anaerobic headspace can be maintained, allowing surface colony growth of anaerobic organisms (Gannon and Thurston 1996). The use of Oxyrase negates the need for anaerobic chambers, inert gas cylinders, or other devices. Oxyrase has also been found to stimulate the growth of injured and uninjured, facultative, and anaerobic organisms (Adler and Spady 1997). Oxyrase has already been used in media fill simulations during asceptic filling line validations.

Immunological Methods

Enzyme immunoassays (EIAs) such as enzyme linked immunosorbant assays (ELISAs) or latex agglutination are widely used in the food industry for rapid screening or confirmation of pathogenic organisms, such as *Salmonella* species, *E. coli O157, Listeria,* and *Staphylococcus aureus.* There is no reason why systems used to detect, for example, *Salmonella, Staphylococcus aureus, Streptococcus* toxins, and *Candida albicans,* cannot be used in the pharmaceutical industry.

EIAs are segregated into competitive and noncompetitive systems, and they reply upon the quantitative reaction of antigens (cell surface proteins or toxins, and so forth) with their anti-body. This interaction is subsequently visualized by various, usually enzymatic means—e.g., fluorescence, chromogen color development.

Immunological methods are useful for screening and detecting specific microorganisms and their toxins. They are quicker than conventional methods, especially if used in conjunction with immunoconcentration techniques, such as IMS. Pre-enrichment is commonly a requirement, because immunological methods require=104 cells/mL to ensure detection. With these requirements, assay times vary from 24 to 52 hours for specific bacteria and 4 to 24 hours for microbial toxins. The major drawbacks are false positives due to nonspecific binding and false negatives in that dead cells are also likely to be detected. Immunological systems are available in a variety of formats, such as microtiter plates, dipsticks, and automated systems. Whatever the format, EIAs provide savings in time and resources. Although pre-enrichment may be required, the preparation and labeling of selective media, plating out, and reading final results can be significantly reduced. In addition, the time to result is shorter. *Salmonella* detection has been a major focus for many of the dipstick and microtiter formats such as TECRA (TECRA Diagnostics, United Kingdom), Ridascreen (R-Biopharm GmbH), TRANSIA (Transia, France), Reveal (Neogen Inc., Ross Labs, United Kingdom), VIP (Biocontrol Systems, United States), Locate® (Rhone Poulac Diagnostics Ltd, United Kingdom), PathStik (Celsis, United Kingdom). The automated immunoassay systems include Vida (bioMérieux, United Kingdom), EIA Foss (Foss Electric, Denmark), and the TECRA Opus (International BioProducts, United States). These methods are discussed further in Chapter 8.

Electrical Methods

Impedance

Growth media for microorganisms comprise relatively large uncharged or weakly charged molecules—i.e., fats, carbohydrates, proteins. Microbial metabolism breaks down the large molecules into smaller, more highly charged components—fatty acids, organic acids, amino acids. These more highly charged molecules cause a change in the electrical conductivity and resistance in the media. Measuring the changes in electrical impedance, capacitance, and/or conductance reveals the organisms in the original sample. The point at which changes in electrical measurements are recognized as a result of microbial activity is known as the time to detection (TTD). This parameter is the time taken at which the number of microorganisms reaches a critical mass that produces a significant change in the electrical activity of the media. This critical mass is usually around 10^6 cells/mL (Cady et al. 1978). Time to detection is directly correlated to the number of organisms in the original sample and also the growth rate of these organisms (Silley and Forsythe 1996). For further information see Chapter 7.

In theory the TTD and activity observed are reproducible if the conditions are exactly the same. Thus serial dilution of the sample with known levels of an organism will provide a standard curve against TTD for which all future samples (of the same material) can be compared. The measured TTD from the sample can give a direct estimate of the initial sample population. This measure can also be used to test materials against a specification as a presence or absence test. Because pharmaceutical dosage forms vary, as do the type and number of microorganisms within samples, it is difficult to develop a single standard curve for all product types. Additionally, it may be difficult to develop a single calibration curve for typical nonsterile pharmaceuticals, unless a large number of samples is used in validating and establishing the standard curve. The difficulty arises because the growth rates and metabolic condition of the microorganism may vary from sample to sample; thus the TTD will change.

The three principal impedance-based systems commercially available are the Bactometer® (bioMérieux), the Malthus® system (Malthus Instruments, United Kingdom), and the rapid automated bacterial impedance technique or RABIT (Don Whitley Scientific, United Kingdom). In many cases the systems have a lot in common.

The advantage of these systems is that they are automated, are simple to use, and have a very high throughput of samples. They can undertake a large number of experiments at any one time, and individual samples can be accessed without disturbing the others. Cost per sample is very low. Despite the drawbacks described above, electrical methods would provide a cost-effective qualitative screening test for pharmaceutical raw materials, intermediates, finished products, and even environmental samples.

An application for which electrical technology is ideally suited is the evaluation of antibiotic resistance and, in particular, preservative efficacy. Connolly et al. (1994) demonstrated that impedance can be used for preservative efficacy testing (PET) of pharmaceuticals and cosmetics.

Bioluminescence

All living cells possess adenosine triphosphate (ATP) as the main chemical energy store. In the presence of the substrate D-luciferin, oxygen, and magnesium ions, the enzyme luciferase will utilize the energy from ATP to oxidize D-luciferin and produce light. (Further details are given in Chapter 6.) This is the mechanism used in the firefly and can be summed up in the following equation:

$$\text{ATP + D-luciferin + } O_2 \text{ - luciferase} \xrightarrow{Mg} \text{AMP + oxyluciferase + } CO_2 \text{ + ppi + light}$$

The amount of light or bioluminescence produced can be measured by sensitive luminometers and is proportional to the amount of ATP present in the sample. The emitted light is usually expressed as relative light units (RLUs). ATP within cells will vary from organism to organism and also with the metabolic state of the microbe; thus many industrial applications are presence or absence tests. However, direct estimate of microbial numbers is possible in certain raw materials, such as raw milk and meat, that are lightly processed and have a relatively high bioburden. Thus direct enumeration in pharmaceutical applications is possible only with some form of sample pretreatment to collect and recover low numbers of stressed organisms.

ATP bioluminescence offers many advantages in terms of speed to result and ease of use. However, because of its lack of sensitivity—requiring 10^3–10^4 organisms for detection— pre-enrichment processes have had to be incorporated into the test regime. In addition, because this is an enzymatic, chemical reaction, it is sensitive to other components within the assay system. For example, quenching or enhancement of the bioluminescent reaction may occur from enrichment broths or from the test samples. Furthermore, samples may also contain endogenous, nonmicrobial ATP, providing false positive reactions.

Fortunately, advances in bioluminescence reagents allow for selection or removal of contaminating ATP. Another disadvantage is that the release of ATP from the cells is disruptive, and therefore further evaluation of any organisms recovered from the sample cannot be performed.

Bioluminescence technology has been particularly useful in the food industry, where the sensitivity constraints are less of a problem. The presence of soil containing microbial and/or nonmicrobial ATP is an indicator of hygiene testing—e.g., Biotrace (Biotrace, United Kingdom), Lightning™ (Biocontrol Systems, United States), Hy-lyte (Glengarry, Biotech, Canada), Charm 4000 (Charm Sciences, United States), and systemSURE (Celsis, United Kingdom). Based upon sensitivity alone, none of these are suitable for pharmaceutical applications. Where they are used, hygiene monitors have often been hampered by incorrect validation of the test system. The Pallchek™ luminometer (Pall Life Sciences, United Kingdom) has been shown to be more sensitive (Davidson et al. 1997). The Pallchek (Whitlock 1994) is a portable luminometer that is capable of measuring surfaces directly as well as swab, filter, and liquid (1–5 mL aliquots) samples within 1 minute. Thus it is potentially possible to perform real time measurements for water quality, air monitoring, direct liquid product monitoring, surface environmental monitoring, and also filterable materials—e.g., raw materials, intermediates, finished products. The Pallchek may be a useful tool to provide a rough index of microbial levels in various aspects of pharmaceutical manufacturing.

The use of bioluminescence for sterility testing in pharmaceuticals has been investigated (Bussey and Tsuji, 1986). The RapiScreen™ (Celsis, United Kingdom) was developed to perform rapid analyses on nonsterile pharmaceutical products.

The RapiScreen system provides a presence or absence test based upon taking a sample and performing 24–48 hours of enrichment. Aliquots from the sample are transferred to cuvettes that are placed within a luminometer. The luminometer adds a permeasing agent to release the ATP from the cells, and then bioluminescent reagents are added. The light produced is measured as RLU, and an RLU value of twice the blank is considered to indicate the presence of microorganisms. The use of pre-enrichment not only provides the right level of organism for detection by bioluminescence, but also provides a culture for further analyses if required. One of the major benefits of this system is the supporting documentation on design qualification (DQ), installation qualification (IQ), operation qualification (OQ), and performance qualification (PQ) of the RapiScreen to meet the demands of the regulatory bodies. This documentation also includes aspects of method validation as described in U.S. Pharmacopeia (USP) Chapter <1225> validation or compendial methods, and it also incorporates the validation elements of the Parenteral Drug Association (PDA) Technical Report No. 33 on validating microbiological methods (see Chapter 11). Morris et al. (1997), and Wills et al. (1997b) described the successful evaluation and validation of ophthalmic solutions, oral drug suspensions, medicated ointments, and cough syrups. We have also evaluated more than 50 materials (raw materials and intermediates and finished products) in the manufacture of antacids, antihelminthics, analgesics, and antiviral materials, covering the dosage forms, liquids, creams, ointments, capsules, and tablets; no material was found to be incompatible with the technology.

Sensitivity of the systems, on the basis of studies to date (Morris et al. 1997), is less than 5 organisms per sample, detectable within 24 hours.

The use of the RapiScreen technology for the routine evaluation and release of a nonsterile pharmaceutical has been approved by the Medicines Control Agency of the United Kingdom (Weatherhead 1997). This is the first regulatory approval of such an alternative technology for use in the pharmaceutical industry.

Millipore (United Kingdom) has used its expertise in filtration coupled with bioluminescence for rapid analyses of filterable samples to estimate total viable material. This MicroStar™ technology counts microorganisms directly on a filter by means of a closed coupled detector camera, and expresses the results as CFUs (colony forming units). It is claimed to be sensitive down to 1 CFU in any sample. The sample is filtered and reagents are sprayed onto the membrane prior to analyses. The detection system is designed to intensify the signal from the microorganisms, and the image processing enumerates the organisms. All images and data are stored and can be downloaded to other computer systems.

Filtration and bioluminescence have also been combined for the evaluation of liquid samples by Celsis in their MicroCount Digital™ system. The system was initially designed for evaluation of the microbiological quality of pharmaceutical waters, and it incorporates most probable number (MPN) statistical evaluations. MicroCount Digital uses a 96 well microtiter plate with a 0.22 µm filter base. This is connected to a four quadrant sample chamber, which allows for four samples to be processed. Each sample thus uses 24 wells on the microtiter plate as the basis of the MPN statistical enumeration.

In 1998 Wills et al. published a stimuli to revision in the USP pharmacopeial forum for incorporation of the Microcount Digital system in the USP testing monograph for pharmacopeial waters. The data provided for this stimuli to revision validated the system, demonstrated equivalence to the standard technique, and showed the equipment capable of detecting 1–10 organisms per 100 mL—i.e., capable of monitoring water for injection (WFI).

Many of the constraints of bioluminescence have been overcome by development of the technology and test protocols. Considerable benefits are to be obtained from the speed to result and the use of resources—e.g., removal of labor-intensive plate reading. However, sample preparation time remains the same as in traditional methods.

Sensitivity of ATP bioluminescence still remains at 10^3 organisms, the reason for a growth or pre-enrichment phase. Squirrel and Murphy (1994) highlighted that sensitivity of the direct ATP bioluminescence reaction could be increased by

coupling to adenylate kinase (AK) activity. AK is an intracellular enzyme, present in all living things, that catalyzes the reaction

$$2\ ADP \rightarrow AMP + ATP.$$

So ATP amplification can occur in the cell through the use of AK. In theory the coupling of the AK and ATP bioluminescent reactions should allow for the detection of a single CFU within 25 minutes. Celsis has been developing the AK technology and will soon market systems that are capable of enumerating, down to a single cell, within 4 hours.

Cytometry

Laser scanning cytometry (LSC) combines membrane filtration, direct viability staining (epifluorescence), and laser scanning. Chemunex SA (France) has developed the ScanRDI®. This system is only for filterable materials but has a sensitivity of 1 CFU per sample with a result in less than 2 hours.

The sample is filtered, labeled, and then laser scanned. Labeling involves the passive diffusion of a nonfluorescent substrate derived from fluorescein (Fluorassure®).

The fluorescein substrate is cleaved by esterase activity in the cell cytoplasm, and its retention within the cell is a function of the organisms cell membrane. Therefore, only viable organisms are detected, on the basis of metabolic activity and an intact plasma membrane. Although labeling devices are available, this part of the sample preparation does require significant manipulation of membranes.

Labeled membranes are transferred to the ScanRDI stage and laser scanned within 3 minutes. The scan lines overlap, so that the whole membrane is scanned. Two photomultiplier tubes (PMT) collect data at specific wavelengths, and the signals recorded are digitally processed to differentiate between labeled microorganisms and background noise (electronic, optical, or autofluorescent particles).

The data analysis removes the background to leave the microorganisms, which are displayed as either a direct cell count or as a scan map. The scan map shows visually the position of each microorganism on the filter. A fluorescent microscope can be linked to the computer of the ScanRDI system, and the computer can control the stage of the microscope. The filter and its holder can be transferred from the ScanRDI to the stage of the fluorescent microscope, and each organism on the filter can be verified visually.

Full documentation, training, and validation support are provided with the ScanRDI.

The obvious benefits of the technology are its speed (result within 2 hours), its sensitivity, and the absence of requirements for growth, avoiding pre-enrichment or selective processes for fastidious or stressed microorganisms. Apart from the potential for faster product release and reduced inventories, the ScanRDI can be a proactive quality assurance system that builds quality into the process and products. It has the potential to identify problems early, allow appropriate re-sampling and testing to verify the situation, and assess rapidly the effectiveness of any corrective action.

Real and potential pharmaceutical applications of the ScanRDI include raw material, in-process and finished product evaluation, pre-sterilization bioburden analysis, preservative efficacy testing, biological indicator maintenance and assessment, microbial contamination control of mammalian cell cultures, environmental monitoring (air, surfaces, personnel), and water testing. The technology is also being developed for sterility testing.

Jones et al. (1999) submitted their evaluation of the technology as a stimuli to revision for inclusion in the USP chapter "Water for Pharmaceutical Purposes <1225>." The system also demonstrated equivalence for both purified water and water for injection, using a multi-site assessment of water samples from several pharmaceutical companies. Chemunex SA is developing techniques to facilitate the identification of microorganisms (nucleic acid probes, antibodies, specific enzyme substrates); they are not yet available.

Chemunex SA has also developed a flow cytometry system, the Dcount®, that detects microbial cells in suspension as they pass through a flow cell. The Dcount incorporates the benefits of the Fluorassure labeling with the digital data capture and analysis used on the ScanRDI.

Dcount is developed to process nonfilterable samples, although there is no reason why filterable materials cannot also be processed. The Dcount also incorporates a robotic sample handling system; once a sample has been taken, the labeling processes and injection for analysis are performed automatically. This significantly reduces sample preparation time and necessary resources.

Thus, like the ScanRDI, Dcount can process samples within 2 hours with a sample throughput of 40–60 samples per hour (depending upon sample volume tested) with minimal operator intervention.

Preliminary evaluation of the system was undertaken by Johnson (1999), using pure culture and product analysis. Pure cultures of *E. coli, Candida albicans, Bacillus circulans, B.licheniformis,* and *Enterobacter cloacae* provided a cumulative correlation of Dcount results to plate count of $R^2 = 0.9919$, with a sensitivity of 10 cells/mL. Series of products were evaluated

—liquid, creams, ointments, syrups—across the product groups antacids, analgesics, and anti-microbials, with no compatibility issues with the technology.

Dcount has the same drawbacks as the ScanRDI in terms of identification of recovered organisms. Despite the lower sensitivity, Dcount potentially offers similar advantages to the ScanRDI in terms of real-time, proactive assessment of pharmaceutical operations, supporting raw material analyses, in-process bioburden assessment, environmental monitoring, and so forth. It could also be used as a screening system: if particulates cause a problem with the flow cell, then sample pre-enrichment and an assessment of the enrichment broth for microbial contamination, could provide a presence or absence test.

MOLECULAR-BASED TECHNOLOGIES

Several DNA molecular-based technologies are being developed primarily for the characterization of microorganisms (Olson 1998). One such system is Gene-Trak® (Neogen, United States), a hybridization technique used for the identification of specific organisms, even within mixed cultures, by targeting 16s or 23s rRNA. Woese (1987) identified 16s RNA as demonstrating phylogenic relationships that could be targeted by labeled DNA probes to identify specific organisms (Stahl et al. 1988). This process is facilitated by the presence of up to 10^4 copies of ribosomal RNA within a cell, although the copy number varies depending upon the metabolic state of the organism. Gene-Trak hybridizes specific DNA probes against the rRNA of the target organism. Approximately 10^5–10^6 cells are required to give a positive result; thus some pre-enrichment phase is required. The protocol is generic, however, so the same methodology can be applied to a wide range of microorganisms. The method takes less than 3 hours and requires only minimal equipment. Another advantage is that considerable information is available for the nucleic acid sequences of rRNA, including those of gram-negative organisms. This means that unique DNA probes can be generated for closely related organisms such as *E. Coli* and *Salmonella.*

Another technique for the isolation and detection of microorganisms from samples is the nucleic acid amplification technology. Polymerase chain reaction (PCR) is the more familiar technique incorporating the enzymatic amplification of target DNA sequences by using a pair of specific primers and a thermotolerant DNA polymerase.

In theory PCR provides a rapid, highly sensitive (single-cell detection) and highly specific method for the isolation and enumeration of bacteria yeasts and molds. In practice, about 10^3 organisms/mlL are required to ensure reliable and repeatable amplification (Fung 1997); thus some pre-enrichment on immuno-magnetic concentration may be required (Shaw et al. 1998).

Because the PCR reaction is so efficient, the large amount of amplified DNA may cause cross-contamination problems that result in false positives, so good analytical technique is required. PCR will amplify any target DNA that the primers bind to, and so there is the possibility of isolating nonviable cells. Finally, PCR is an enzymatic reaction, and it is possible that components of the sample may interfere with the amplification (Vaneechoutte and van Eldere 1997). Inhibition can be overcome by dilution or quenching of the inhibiting components (Kreader 1996).

Qualicon (United States) has developed a commercial kit for the detection of food-borne organisms, including *Salmonella* (Sobities et al. 1996), known as Bax® using PCR. Jimenez et al. (1998) used Bax *Salmonella* kits to test raw materials and nonsterile pharmaceutical finished products. They concluded that it was possible to screen the 25 materials tested for *Salmonella* within 30 hours, rather than the usual 5–7 days.

Other quantitative nucleic acid-based amplification assays include Taqman® (PE Applied Biosystems, United States), Ligase Chain Reaction (LCR) (Abbott Laboratories, United States), branched chain DNA (bDNA) assay (Chirons Diagnostics, United States), reverse transcriptase-PCR (RT-PCR, or COBAS™ system), (Roche, United States), nucleic acid sequence-based amplification (NASBA™ system; Organon Teknika, United Kingdom) and Q-beta replicase (Public Health Research Institute, United States). These are also discussed in detail in Chapter 9.

The 16s rRNA key to bacterial phylogeny discovered by Woese (1987) has been utilized in two genetically based technologies, Microseq™ (PE Applied Biosystems, United States) and the RiboPrinter (Qualicon).

Microseq 16s rRNA gene kit identifies bacterial species on the basis of sequences of their 16s rRNA genes. Genomic DNA is extracted and PCR is amplified and then sequenced. A comparison of the sequence information with the 16s ribosomal DNA sequence database allows for definitive identification and for establishing phylogenetic relationships. The level of sequencing performed determines the level of detail required. Advantages of the system are that growth and cultural conditions are unimportant and do not influence the identification, the results always being reproducible. The current drawback to the system is that the sequence database is not extensive. For a review of the technology, see Sasaki et al. (1997) and Chapter 9.

The RiboPrinter is a microbial characterization system that does not use PCR or DNA sequencing (Sethi 1997). From a single colony on a plate, microorganisms are transferred to the automated RiboPrinter. The microbial DNA is extracted, fragmented by endonucleases, electrophoretically separated, labeled with a chemiluminescent substrate, and visualized with a CCD camera. The result is a unique banded pattern of stained fragments, or Riboprint.

Banding patterns are highly conserved at the species and gene level (Bruce et al. 1995, Hubner et al. 1995), and so if the database contains RiboPrint patterns for known organisms, then an identification can be made or a phylogenetic relationship established. The most powerful application of this system is the ability to pinpoint contamination sources (Pfaller et al. 1996). The RiboPrinter can process 32 samples per day and is easy to use. An initial constraint was the organism database; however this is being expanded, facilitated by the ability to network RiboPrinters. More information can be obtained by using different or multiple restriction enzymes during DNA digestion. However, the use of different enzymes does require the development of new databases.

Although systems such as Microseq and RiboPrinter are more definitive phylogenetically, it will be some time before the established biochemically based identification systems are replaced, because the latter are intimately instilled within many pharmaceutical Quality Assurance operations.

IDENTIFICATION SYSTEMS

Identification of microorganisms is very important for all aspects of pharmaceutical manufacturing control: utilities (e.g., water), raw materials, in-process samples, and finished products. Prior to the advent of genetically based technologies such as Microseq and RiboPrinter, most systems relied upon the use of metabolic activity to identify organisms. These "rapid" identification techniques have been quickly adopted by most pharmaceutical microbiology laboratories. A large number of these systems are available, e.g., Micro-ID® (Organon Teknika, United States), API (bioMérieux SA, France), Minitek™ (BBL Microbiology Systems, United States), Enterotube (Roche, United States), Crystal™ (Becton-Dickenson, France), IDS RapID Systems (Innovative Diagnostic Systems, United States). Some systems have become automated, e.g., Vitek (bioMérieux SA, France) and Biolog Omnilog (Biolog, United States). The systems vary in their level of hands-on requirements and means of interpretation of results, although automation has improved these areas. Most systems were developed around clinical or food pathogen isolates, but manufacturers have expanded to support the identification of nonfermenters, anaerobes, Gram-positive organisms, yeast, and molds.

Gas chromatography is another means of identification. Bacteria and fungi have been shown to be identifiable to strain level on the basis of cellular fatty acids (Moss 1981, Olson 1998). The commercial system, called MIDI (MIDI Inc., United States), relies upon saponifying fatty acids from cells from a culture. The fatty acids are then extracted and injected into an automated gas chromatography unit. The resulting chromatogram is compared to an established database to aid identification. The advantages of the system are that the number of fatty acids is high (>300), and therefore a unique sequence is found for individual organisms. Similarly, the analysis is reproducible, even from laboratory to laboratory, and the automation minimizes operator input. However, consideration must be given to standardization of cultural conditions (temperature, media type) and also to the age of the test culture, because these factors will influence fatty acid composition.

CONCLUSIONS

Microbiological methods in the pharmaceutical and medical device industries have remained static for most of this century. The changing environment within the healthcare market is bringing pressure to bear on traditional techniques. Advances in rapid methods and a greater understanding of the pharmaceutical sector by manufacturers are beginning to have an impact.

There clearly are rapid microbiological technologies that are faster, cheaper, and more sensitive than traditional nineteenth century techniques. However, no single technology is currently capable of providing all desirable traits. For example, sensitivity and speed would not necessarily result in a cheaper technology. It is therefore essential that the testing required is evaluated and used to produce a business case for the attainment of any suitable rapid microbiological system. Some suppliers —e.g., Celsis—provide support in this financial justification area.

It is also important that the application of the technology to any specific activity is understood, so that appropriate validation can be undertaken. A rapid "in-process" screening method may be easier to validate than a finished-product release test. Additionally, validation requires a thorough understanding of the technology. Suppliers are now providing support for validation of new microbiological methods and are also driving a process to educate regulators in the new technologies.

The approval and acceptance of rapid methods into the pharmaceutical sector is a slow process. It requires a great deal of time, commitment, and money. Introduction of new methods is not, however, impossible; examples such as the introduction of the LAL (*Limulus* amebocyte lysate) test for bacterial endotoxin testing, automated identification systems, and ATP bioluminescence for nonsterile product testing are examples of what is possible.

The potential rewards for technology suppliers, pharmaceutical manufacturers, and patients are very high. Introduction of rapid methods into pharmaceutical microbiology has only just begun. As the process of introduction becomes clearer and precedents are set, the pace of change will accelerate.

The technologies of the nineteenth century are finally giving way to new methods. The new technologies currently being developed will fundamentally change the way in which pharmaceutical microbiology is performed well into the century. It

will be interesting to see if these emerging technologies stand the test of time as successfully as the classical methods developed by the nineteenth century pioneers of microbiology.

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5
Labor-Saving Devices and Automation of Traditional Methods

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Speed of analytical result is often less important than ability to test large numbers of samples, carry out tests "in the field," or obtain "images" of microbial contamination when there are problems. This chapter will briefly describe techniques that save technical time, compared with traditional methods. Several venerable techniques are remarkably efficient and should not be overlooked if the situation calls for more testing than the laboratory can normally handle, or when unusual problems of contamination have not been pinpointed by conventional methods.

BLENDERS, STOMACHERS, AND PULSIFIERS

A common first step in microbiological analysis is to suspend microbes from the sample in a suitable diluent; bladed or turreted blenders have been the traditional means to prepare the initial suspension in many areas of microbiology. Problems of noise, the need to clean and sterilize after use, overheating, and high levels of debris in suspensions led to development of the Stomacher® (Seward Medical, United Kindgom), which processes samples in disposable plastic bags, massaging and crushing them under two paddles. It causes less tissue disruption than do bladed blenders, and smaller temperature rises in the suspension (Sharpe and Jackson 1972). After expiration of the patents, at least five instruments similar to the Stomacher appeared on the market, and the generic instrument is now better referred to as the paddle-action processor.

The Stomacher was seen to be very convenient, but suspicion about its less destructive action led to many performance evaluations. Because it was originally developed for food microbiology laboratories, the literature on paddle-action blenders is heavily biased toward this type of commodity. The original evaluation (Sharpe and Jackson 1972) used peptone water diluent, but following further study (Sharpe and Harshman 1976) addition of 1% Tween® 80 surfactant was recommended to improve microbial yields from samples containing appreciable levels of fat. Because the paddle blender causes less tissue destruction than a bladed blender, suspensions contain less particulate material; this has been an advantage in techniques such as membrane filtration (Sharpe et al. 1979).

In 8 studies the Stomacher yielded about the same count as a bladed blender, in 6 there was no difference, and in about 20 the count was slightly lower. Stomachers gave higher counts than blenders for poultry carcasses (Adams et al. 1980), for cheeses (Asperger and Brandl 1978), for aerobes and gram-negative bacteria from frozen ground beef (Jay and Margitic 1979), in impedance measurements with meats (Bulte and Reuter 1984), for *Trichinella* in pork (Lotzsch 1977a,b), for hard and soft cheeses (Nanni 1975), for meats, seafood, and cooked foods (Tuttlebee 1975), and for lung smears of *Pneumocystis carinii* (Thomson et al. 1982). There were no significant differences between Stomachers and blenders for casein (O'Connor 1976); for ground beef, celery, and wieners (Schiemann 1977); for counts of bacteria on chicken skin (Thomas and McMeekin 1980); for *Trichinella* (Thomsen 1976, 1977); for highfat dairy foods (Thrasher and Richardson 1980); and for counts of *Listeria monocytogenes* from Colby cheese (Yousef et al. 1988). The Stomacher gave slightly lower counts of coliforms and *Salmonella* in fatty foods (Andrews et al. 1978b), of *Salmonella* from frog legs (Andrews et al. 1977), in a general comparison (Andrews et al. 1978a), in meat products and salads (Baumgart 1973, 1977, 1980), from raisins, hard cheese, and wheat grains and for molds in meat and sausages (Blaser 1978), for counts from human colon walls (Croucher et al. 1983), of *Bacillus cereus, Yersinia enterocolitica,* and *Staphylococcus aureus* (Deibel and Banwart 1982), of *Listeria monocytogenes* in beef tissues. Although Tween 80 increased the number of bacteria recovered from fat tissue (Dickson 1990), in meats, with or without Tween 80 (Emswiler et al. 1977), of trichinae (Framstad (1978), of *Clostridium perfringens* (Harmon and Kautter 1979), from chicken carcasses (Klinger et al. 1981), of aflatoxin in egg powder (Lotzsch et al. 1974), from sputum (Mitchell and Harvey 1975), of Enterobacteriaceae in chicken (Mulder 1975), and in seafood, pork, ham, and

sausage (Niwayama et al. 1975). General reviews of Stomacher performance can be found in papers by Gerats and Snijders (1977a, 1977b, 1978), Goldschmidt and Fung (1978), Harrigan and McCance (1976), Lotzsch et al. (1974), Lotzsch (1977a,b), Richardson (1981), Sharpe and Jackson (1972), and Sperber (1976), while others cover safety aspects (Collins et al. 1974), improved bags with filters (Konuma et al. 1982), and control of foaming (Stersky et al. 1980).

A recent advancement over the paddle-type processor is the Pulsifier® (Microgen Bioproducts Ltd., United Kingdom). This instrument has several attractive features. Instead of kneading and crushing samples, it applies a combination of shock waves and intense agitation. It also accepts samples in disposable plastic bags. Because microbes usually exist on surfaces or within easily accessible structures, "pulsification" efficiently disperses them. In a comparative trial (Fung et al. 1998) based on total aerobic counts from 96 samples of representative foods, the average ratio of Pulsifien: Stomacher was 1.4. This high ratio resulted mainly because counts by Pulsifier at very low count levels were approximately twice those obtained by Stomacher, while at count levels above $<10^5$ CFU/g the difference disappeared. Coliform counts from 85 food samples and *Escherichia coli* counts from 36 samples indicated no significant differences between the Pulsifier and a paddle-type processor (Sharpe et al. 2000), although, in each case, counts at low levels tended to be higher from the Pulsifier. Improved counts at low levels probably reflect reduced interference and better visibility of colonies, because pulsified materials contain less food debris. For celery and carrot, Pulsifien: Stomacher total count ratios were 1.3 and 2.5, respectively; Pulsifier suspensions were clear, however, whereas Stomacher ones contained enough debris to interfere with pipetting (Fung et al. 1998). Improved membrane filterability (for example, filtration speeds of 12:1 and 10:1 for beef liver and broccoli, respectively, down to 1.4:1 and 1.3:1 for ground chicken and feta cheese, respectively), reflect the reduced levels of suspended material by the Pulsifier (Sharpe et al. 2000). This may prove beneficial for techniques in which interference by sample components is a problem (e.g., polymerase chain reactions or ATP bioluminescence). In fact, even counting conventional agar plates is facilitated by reduced debris levels in or on the agar. Because the Pulsifier does not crush samples, hard objects cause less damage to the bag. A removable, transparent door allows the action to be viewed and improves accessibility for cleaning.

GRAVIMETRIC DILUTERS

In most facets of microbiological work, the requirement for sample dilution is inevitably labor intensive. In general terms the initial sample needs to be aseptically weighed and the precise volume of diluent added. It is common to prepare tenfold serial dilutions for purposes of microbial enumeration.

The principle of gravimetric dilution was established in 1982; the first systems targeted the food industry. In the original patent, the gravimetric diluter was described as an apparatus for making dilutions of a sample with a diluent to a predetermined dilution factor. In essence the system is made up of a top pan balance or load cell connected to a pump with a programmable user interface.

The user is able to program the instrument such that the sample is automatically weighed and a predetermined volume of diluent added to give the requisite dilution. Manninen and Fung (1992) showed that it was possible to provide accuracy of ±0. 2 g in delivering a fixed weight of diluent to a weighed sample. The current generation of gravimetric diluters dispense diluent to within 1% accuracy of the calculated final weight, are able to accommodate sample weights in the range 1 g to 1, 500 g, and can achieve 1,000-fold dilutions, depending upon the initial sample weight. The time taken to achieve such dilutions is extremely fast: weighing and diluting can be completed within 30 seconds/sample by utilizing pump speeds of up to almost 1000 mL/minute. Compliance with GLP (Good Laboratory Practice) is supported through the ability to connect to an external printer, thereby providing a hard-copy record of the completed dilution.

COUNTING PROCEDURES

When the sample suspension has been prepared, it will usually be plated, incubated, and counted. Several variants of traditional methods save labor, materials, or both.

Hydrophobic Grid Membrane Filter

The hydrophobic grid membrane filter (HGMF) (Sharpe and Michaud 1974; Sharpe and Peterkin 1988; Sharpe 1989) combines features of plate, membrane filter (MF), and most probable number (MPN) counts. Its unique properties result from the confining of colony growths to the grid-cell in which they originated; a typical appearance after incubation is of a grid carrying a random distribution of square "colonies." The HGMF was developed by the Canadian Health Protection Branch as a vehicle for automated counting. The ISO-GRID™ HGMF, developed by QA Lifesciences Inc., is available from Neogen (United States). In addition to techniques for total aerobes, many techniques for detection of pathogens have been developed.

Sample preparation consists of dispersing by Stomacher, digesting with an enzyme if necessary, vacuum filtering, and laying the HGMF on a growth medium. Serial dilutions are usually unnecessary. A unique apparatus called Spreadfilter

(Filtaflex Ltd., Canada), relies on the hydrophobic border of the HGMF to retain sample volumes up to 10 mL. One uses the pipette to dispense and spread the sample over the HGMF. The rotatable filter head makes this easy. The Spreadfilter can be immediately reused without sterilization. Automated counts of HGMFs can be made using the computerized HGMF Interpreter (Filtaflex Ltd., Canada). This inexpensive computer attachment counts any colonies on an HGMF that are similar to one initially indicated by the technician by means of the computer mouse. The HGMF Interpreter also records (JPEG) files of the HGMF images, giving users the security of knowing they can reinspect an HGMF months after the analysis was done. This capability could be of considerable value in cases of dispute over the microbiological findings.

The HGMF has unique advantages in the enumeration of organisms (e.g., removal of inhibitors or unwanted nutrients, concentration of organisms, transferability across media to aid resuscitation, and wide [10^4:1] linear counting range). These properties are less evident during pathogen detection, since an enrichment stage is usually required. However, there are several other attractions; described below.

There are HGMF-based analyses for most common microorganisms; this permits a unified approach to laboratory procedures. Many HGMF techniques (aerobic plate count, coliform, fecal coliform, *E. coli,* and *Salmonella, Escherichia coli* O157:H7) have been designated AOAC (AOAC International) Official Methods or are described in the *Compendium of Methods for the Microbiological Examination of Foods* (Vanderzant and Splittstoesser 1992). Sharpe and Peterkin (1988) reviewed most HGMF and other membrane filtration procedures, including those for fecal streptococci, *Staphylococcus aureus, Vibrio parahaemolyticus, yeastsandmolds,* Clostridium *perfringens, Pseudomonas aeruginosa,* and *Yersinia enterocolitica.*

Replicate HGMFs (made easily and quickly) can be incubated on different media so that a biochemical profile can be obtained for the growths in each grid-cell. As many as 400 different cultures have been maintained on a single master HGMF (Sharpe et al. 1989a, 1989b). HGMF Replicators (Filtaflex Ltd.) have been used in various studies, for example, the prevalence of antibiotic resistances in *E. coli* among swine (Dunlop et al. 1998a,b) and prevalence of *shigatoxigenic E. coli* (STEC) in dairy herds (Cobbold and Desmarchelier 2000).

Growths on membrane filters can be subjected to complex staining and identification reactions that are impossible on plates. This property is exploited in a range of detection procedures.

Analysis cost by HGMF compares favorably with other techniques. For example, Chain and Fung (1991) estimated that the cost of doing aerobic plate count (APC) by ISO-GRID HGMF was U.S. $3.33, compared with $13.62 by standard APC, and $8.22 by both Petrifilm™ and Redigel™. Massive financial savings were found when the HGMF system was used to investigate microbial loads in abattoirs, as part of the Canadian Food Safety Enhancement Program. Laboratories based on HGMFs set up directly on abattoir floors to study HACCP (Hazard Analysis Critical Control Point) in poultry plants were supplied agar plates containing triphenyltetrazolium chloride (TTC) and the diluents. Chickens were rinsed in plastic bags in a commercial paint shaker handling six samples at a time, and returned to the line. Rinses were filtered onto HGMFs by using a Spreadfilter, and the HGMFs were plated on the TTC plates. After incubation the HGMFs were read in the computerized Interpreter by the inspector or factory staff, who simply put the petri dish into the instrument and recorded the result. The cost of counting birds this way was *one-tenth* that of sending them to the lab; in one study alone, more than 6,000 birds were tested (McNab et al. 1991). Repeatability of the analyses was excellent, and because they were done on the spot, the HGMF results were highly pertinent. Similar studies were carried out in beef abattoirs to determine beef carcass contamination (Charlebois et al. 1991; Fliss et al. 1991).

Petrifilm

Petrifilm™ and Redigel™ are examples of commercial products designed to reduce the manipulative work in counting bacteria. An advantage of Petrifilm (3M Microbiology Products, United States) is its simplicity. No medium preparation is needed, and sample handling is simple and fast. Aseptic technique is required only while inoculating. The method is useful if labs are overwhelmed by media production and sample preparation, or have limited incubator space. A benefit claimed for the enumeration of microorganisms by the Petrifilm method is the avoidance of temperature stress caused by molten agar. The analytical aliquot is added to a dry culture medium coated on a 20 cm^2 film base. The bottom film containing growth medium is printed with a counting grid of 1×1 cm squares, and overlaid with a polyethylene film coated with water-soluble gelling agent. To use Petrifilm, the cover of the film is raised and the surface inoculated with 1 mL of sample. The cover film is replaced, and a plastic spreader applied to the outside of the film to ensure dispersal of the sample; the gel solidifies within 1 minute. Petrifilm plates are incubated at conventional temperatures and times. Comparisons of Petrifilm performance with conventional plate methods generally are favourable for Petrifilm.

Standard Plate Count

The medium for Petrifilm SM contains TTC dye, which yields red colonies for TTC reducers. Petrifilm SM is an AOAC International Official Method based on data for milk samples (Ginn et al. 1986). While the Petrifilm method gave slightly lower recoveries than other plate methods, the repeatabilities and reproducibilities were not significantly different. A comparison of Petrifilm with other aerobic plate count methods such as Redigel, ISO-GRID, spiral plater, and APC for various foods (Chain and Fung 1991) indicated that for ground beef or pork and raw milk, counts were statistically identical to and highly correlated with the aerobic plate count method. Petrifilm and Redigel both cost $8.22 per test, compared with $13.62 for the APC, $3.33 for ISO-GRID, and $2.27 for spiral plater.

Coliform and Escherichia coli

Coliform colonies also produce red colonies by reduction of TTC, and gas produced by lactose fermentation accumulating around colonies aids their differentiation. Petrifilm E. Coli Count (EC) plates contain the same ingredients as Petrifilm Coliform Count (CC) plates, plus a glucuronidase activity indicator, 5-bromo-4-chloro-3-indolyl-D-glucuronide (BCIG). Glucuronidase activity results in formation of indigo-blue colonies; the glucuronidase-negative colonies remain red. Only blue colonies with gas formation are counted as *E. coli.* Glucuronidase activity is moderately specific (90–100% of strains) to nonpathogenic *E. coli* strains (e.g., Feng and Hartman 1982; Koburger and Miller 1985; Petzel and Hartman 1985; DeLisle and Ley 1989; Rippey et al. 1987; Sharpe et al. 1989a). About 20% of *Salmonella* strains and 30% of *Shigella* and *Hafnia* strains are also glucuronidase positive (e.g., Feng and Hartman 1982; Damare et al. 1985; Sharpe et al. 1989b). Occasional glucuronidase strains of other Enterobacteriaceae have been reported. Petrifilm CC can be used only for coliform counts; Petrifilm EC can be used for both coliform and *E. coli* enumeration.

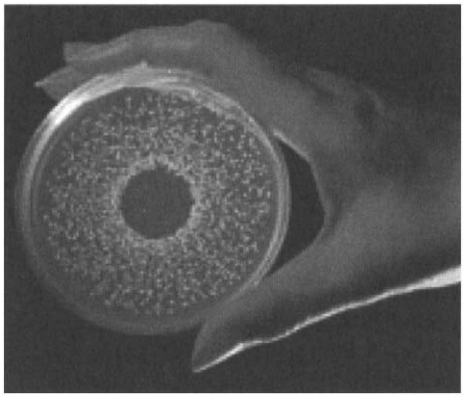
For raw milk samples (Nelson et al. 1984), Petrifilm EC VRB counts were about 96% of those from a plate method using VRB agar, and about 78% of those using MPN methods with lauryl sulfate and brilliant green lactose bile broths. Petrifilm EC is an AOAC International Official Method, based on data for milk samples (Ginn et al. 1986). Comparisons were made against standard plate count and VRB agar plate counts. While both Petrifilm methods (CC and EC) gave slightly lower recoveries than the plate methods (0.027 and 0.013 $\log_{10}$, respectively), the repeatabilities and reproducibilities of all methods were not significantly different. However, in one study on poultry samples, while Petrifilm EC gave virtually equivalent counts for the total count method, problems of reading Petrifilm EC plates, caused by the background flora, precluded making a coliform count comparison (Bailey and Cox 1987). If coliforms were less than 10% of the total population, it was difficult to count the gas-producing colonies.

Coliform recoveries by Petrifilm EC were deemed comparable to a VRB plate method, and Petrifilm *E. coli* counts tended to be higher than counts by the AOAC International MPN *E. coli* method, at both 24 and 48 hours, for cheese, vegetable, and poultry samples (Matner et al. 1990). In comparing Petrifilm EC to the Standard Methods for Examination of Dairy Products (Richardson 1985) and the AOAC International method (Curiale et al. 1989) for detection and enumeration of coliforms and *E. coli* in ice cream and ice milk, Matushek et al. (1992) found that direct plating of the samples was less reliable than plating a diluted product. This lesser reliability may have been a result of the presence of fermentable sugars or substances such as chocolate, which may be inhibitory or cause atypical growth; Petrifilm EC method required 24–48 hours, depending on the sample, and for 5 of the 6 products tested, the method gave higher confirmation rates (94–100%) than the other methods. Slabyj et al. (1991) found only a low correlation (0.6) between counts of fecal coliforms in seafood processing plants, counted by MPN, Petrifilm EC, and Redigel VRB methods at 44.5°C. Because of the low fecal coliform counts, Slabyj et al. favored the MPN method because of its greater sensitivity. Finally, the Petrifilm EC method tended to give lower counts but better repeatability than the AOAC International MPN method for a variety of foods and was accorded AOAC Official Method status (Curiale et al. 1989).

Petrifilm E. coli O157:H7

The Petrifilm kit to detect *E. coli* 0157:H7 is based on a blot-ELISA technique. Enriched samples are plated on Petrifilm EC plates and incubated at 42°C for 18 hours; colonies are blotted to a reactive disk for 2 minutes, and the disk is then washed and reacted with an antibody-enzyme conjugate for 30 minutes. After further thorough washing, the disk is incubated 5–10 minutes in enzyme substrate and dried. Positive colony blots are dark gray to black; the disk is compared to the original, so that suspect colonies can be picked off for further identification. The antibody cross-reacts with *Salmonella* type N (rare), some *Yersinia* O9, and all *E. coli* O157 whether H7 or not, and possibly with *E. coli* O7 and O116. The 3M company provides an isolation protocol for the organism from raw hamburger.

Figure 5.1 Typical pattern of colony distribution on a spiral plate.



Yeast and Mold

For selected dairy products and high-acid foods, correlation coefficients for the Petrifilm yeast and mold (YM) method were 0.993 to 0.995 compared to surface and pour-APDA and CPCA plates, Petrifilm offering an acceptable alternative to traditional methods (Beuchat et al. 1990). While correlation coefficients were 0.961–0.974 compared to APDA and CPCA, particle interference was found to make enumeration of colonies difficult for some foods, because food particles also developed the blue color (Beuchat et al. 1990, 1991).

Redigel

Redigel® (Neogen, United States) uses a low-methoxyl pectin combined with Ca^{++} to form a gel. It is provided in units that include liquid media containing nutrients, the gelling agent, and the petri dishes, which contain a thin layer of hardener ($CaCl_2$) that causes the gelling agent to solidify at room temperature. It is very similar to aerobic point or other counts, except that instead of inoculating the plate prior to addition of the gelling agent, in Redigel the liquid nutrients are added first. Solidification time is approximately the same as for APC.

In two publications APC and Redigel counts on foods were reported to show an overall correlation of r=0.964 (Fung and Chain 1991); the costs of Redigel tests equaled those of Petrifilm tests and were higher than ISO-GRID HGMF or spiral plater (Chain and Fung 1991). Slabyj et al. (1991), however, found only a low correlation between fecal coliforms in seafood processing plants counted by MPN, Petrifilm EC, and Redigel VRB methods at 44.5°C. Because of the low fecal coliform counts, these authors favored the MPN method because of its greater sensitivity.

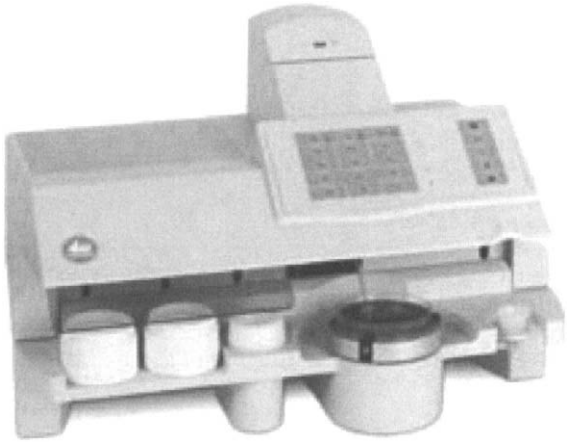
Spiral Platers

Agar pour plate procedures are routinely used for the quantitative determination of microbial loading in a given sample. The technique was introduced by Robert Koch in 1880 with the development of agar culture media and by 1895 was a recognized procedure. In 1916 Breed and Dotherer formalized the procedure in which an unknown sample is serially diluted and a known volume of each dilution mixed with the molten agar in a petri dish. After incubation, plates with between 30 and 300 colonies are counted and the resultant count multiplied by the appropriate dilution factor to obtain the requisite count per milliliter in the original sample.

Such methods are labor intensive and costly in materials; a major part of bench time is spent in preparation of dilutions and inoculation onto the culture medium. A lesser but still significant resource needs to be allocated to preparation of sterile diluent and culture media prior to analysis. A further disadvantage is in the repetitive nature of the work; therefore, alternative approaches to plate counting have been introduced. Trotman (1971) devised a system that mechanically inoculated the surface of an agar plate, but it was not quantitative. A device more suited to the needs of the microbiologist wishing to enumerate microorganisms was introduced by a group from the U.S. Food and Drug Administration (FDA) laboratories in Cincinnati (Gilchrist et al. 1973). The basic concept of the instrument is to continuously deposit a volume of undiluted sample on the surface of a rotating agar plate. The resultant track is in the form of an Archimedes spiral. The sample volume is controlled and decreased as the dispensing stylus is moved from the center to the edge of the rotating agar plate. During incubation, colonies develop along the lines where the sample was originally deposited. The number of colonies per unit length of deposition or per unit area of the agar surface depends on the bacterial concentration in the deposited sample (see Figure 5.1). Because of the different densities from the center to the edge of the plate, some area of each plate can easily be counted. The

Figure 5.2 Spiral plater counting grids.

Figure 5.3 Example of a new generation spiral plater (WASP).



Photograph courtesy of Don Whitley Scientific Ltd.

original patented system allowed the estimation of microbial concentrations over a range of three orders of magnitude from 600 to 6×10^5 CFU/mL without the need for serial dilutions and using only a single agar plate for each sample. The sensitivity of the current generation of instruments, which are all controlled by software rather than mechanically, has been increased by allowing an increased inoculum volume, so that counts as low as 30 CFU/mL can be determined (Table 5.1) over the range 30 to 4×10^5 CFU/mL

Counting

Counting tables are available that relate the number of colonies counted within the requisite sector to a calculated number of colony forming units per milliliter in the applied sample.

After plates have been incubated, they can be counted manually or automatically (see Electronic Colony Counters below). For manual counts the plate is centered over a counting grid as shown in Figure 5.2.

The primary markings are the three concentric circles and the eight radial lines that create annular segments. The grid is further divided by secondary arcs and radial lines that create marked areas. Every marked area corresponds to a known, constant volume of sample deposited on the spiral plate.

The spiral plate method is recognized by the American Public Health Association and is an AOAC International Official Methods. Gilchrist et al. (1977), Jarvis et al. (1977), and Kramer et al. (1979) showed the value of the spiral plater technique relative to the standard plate count.

The original spiral plater, developed by Dr. J.E.Campbell in 1973, was introduced as the Model A; subsequent derivatives were known as Models B, C, and D. Each of these instruments used a cam-activated syringe to supply a liquid sample to the agar plate. The current generation of instruments has taken advantage of contemporary technology by using microprocessor-controlled stepper motors to further automate the original technology. With the exception of the Eddy Jet product from IUL, which uses a disposable tip, the

Table 5.1 Determination of the Lower Sensitivity Level of WASP in the 200 µL Deposition Mode

	n	CFU/mL	SEM
Standard Pour Plate Count	5	31	3.2
WASP Count	5	30	5.2

other instruments, WASP (Don Whitley Scientific, United Kingdom) and Autoplate 4000™ (Spiral Biotech, United States), retain the use of a backfill syringe to ensure a reproducible dispensed volume.

The new-generation spiral platers (see example in Figure 5.3) provide for variable volume of sample deposition. Tables 5.2 and 5.3 show reproducibility data from three deposition volumes offered on the early WASP instruments.

Table 5.2 Reproducibility of Counts from the Standard Pour Plate Method and WASP Using 50 µL, 100 µl, and 200 µl Deposition Modes

Standard Pour Plate Count (CFU/mL)	SEM	WASP Count (CFU/mL)					
		50 µL Deposition		100 µL Deposition		200 µL Deposition	
		Mean	SEM	Mean	SEM	Mean	SEM
$1.1x10^4$	706	1.2×10^4	510	1.1×10^4	245	1.4×10^4	1400
5.9×10^3	288	7.7×10^3	759	6.2×10^3	339	6.3×10^3	383
4.0×10^3	283	4.6×10^3	615	4.2×10^3	171	3.8×10^3	153
3.2×10^3	258	3.1×10^3	103	4.3×10^3	255	4.8×10^3	103
2.1×10^3	308	2.4×10^3	114	3.3×10^3	480	2.9×10^3	132
1.1×10^3	105	1.0×10^3	43	9.8×10^2	55	1.1×10^3	65

Electronic Colony Counters

The use of methods that result in formation of colonies requires that eventually those colonies be counted. Many "conventional" electronic colony counters are available—e.g., Automatic Colony Counter® (Fisher Scientific, United States); BioFoss® (AS/N Foss Electric, Denmark); 3M Model 620 (3M Company, United States); Artek® (Artek System Co., United States); Biotran® (New Brunswick Scientific Co., United States), ProtoCOL® (Don Whitley Scientific, United Kingdom), CASBA 4®, (Spiral Biotech, United States). Early interest was in milk counts, and studies showed that if milk plates were manually screened for suitability, the instrument data met the standards of Standard Methods for the Examination of Dairy Products (e.g., Goss et al 1974; Packard and Ginn 1974; Fleming and O'Connor 1975; Fruin and Clark 1977). All such instruments require calibration for the particular run of plates being examined; changes of colony size, growth medium, or illumination necessitate recalibration. Under less than ideal conditions, where spreaders, overlapping or pale colonies, food debris, opaque media, and so forth are encountered, their reliability drops dramatically. Unfortunately, such conditions are common in microbiological analysis.

Different approaches are found in laser counters for spiral platers and in the HGMF Interpreter. A recent development from the instrument described by Sharpe et al. (1986), the HGMF Interpreter (see "Hydrophobic Grid Membrane Filter," above) is largely immune to the aforementioned sources of error as it does not actually count colonies. It is a property of the HGMF that one does not need to distinguish and count "colonies"; one needs only determine whether each HGMF grid cell does or does not contain growth of the target organism. Thus a grid cell is count "1" whether it contains 1 or 20 individual growths that (on a conventional petri dish) would be counted as individuals; the Most Probable Number mathematic on which the HGMF is based permits accurate counts to be derived from the grid-cell count. The HGMF Interpreter simply calculates the positions of all HGMF grid cells and then inspects

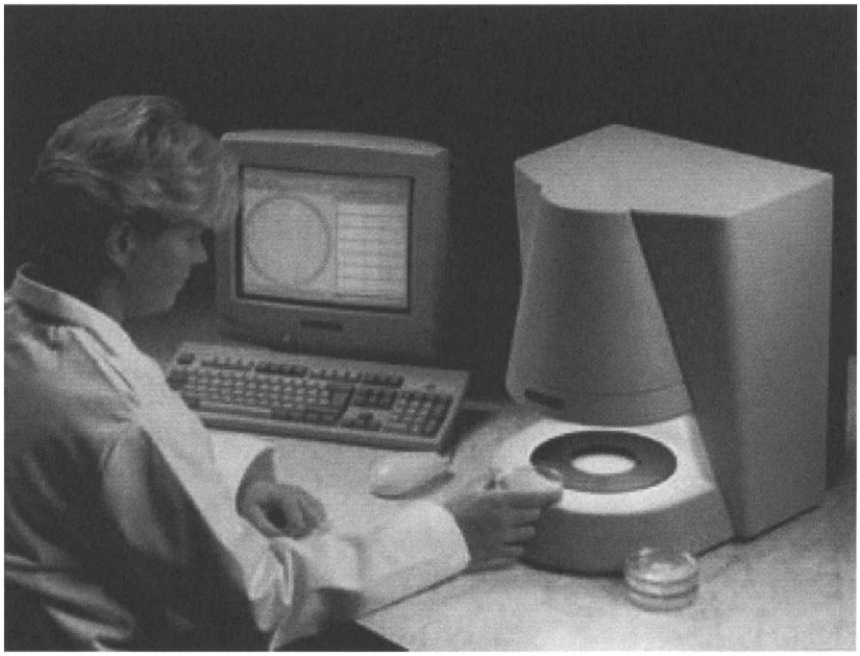
Table 5.3 Reproducibility of the Dispensed Volume of the WASP in 50 µl, 100 µL, and 200 µL mode

	50 µL	100 µL	200 µL
Number of Observations	24	12	12
Range of Dispensed Volume (µL)	50–51	101.0–102.1	197.0–204.9
Mean Volume Dispensed (µL)	50.7	101.7	201.6
Standard Error of the Mean (SEM)	0.04	0.08	0.73

each one for growth similar to a target indicated by the user. In most cases its initial conclusion is satisfactory; however, by changing the range of included color hue or using the Shift or Ctrl keys together with the mouse buttons the user can fine tune the interpreter's results if desired (for example, growths some times invade neighboring HGMF grid cells, which could lead to overcounting). The HGMF Interpreter also allows the saving of images of analyzed HGMFs on the computer's hard drive; these provide users with the security of accurate records for further reference, and are easily transmitted between laboratories.

There are many disadvantages related to the plate count technique; we consider here only those particularly relevant to the case of counting the resultant colonies. The primary problem is that growth and distribution of the colonies on the surface of the agar plate are rarely homogenous. Even with pure cultures, colonies will not always have the same diameter, and density and even shape and growth can often be partly or totally confluent. Mixed culture growth will certainly lead to considerable differences in the above factors, all of which will tend to reduce the accuracy of the count. Manual counting can address these problems, but for laboratories with a high throughput of samples it is not always possible or economically viable; thus, automation needs to be utilized.

Figure 5.4 Example of a typical electronic colony counter (ProtoCOL).



Photograph courtesy of Don Whitley Scientific Ltd.

The challenge for automated counting has been to develop a suitable counting algorithm to meet the needs of the many and varied colony types. Developments in computing have led to the proposal of several different algorithms and counting systems (García-Armestro et al. 1993; Mukherjee et al. 1995; Nishijima et al. 1983; Pickett and Welch, 1995; Spadinger and Palcic 1993; Walsh, 1994; Wilson 1995). Corkidi et al. (1998) presented an image analysis method entitled COVASIAM which, unlike the previously mentioned approaches, could allow detection of individual colonies growing to confluence and could also address the problem of colonies of various sizes. This method uses the light-reflecting optical properties of the surface of most microbial colonies. Most colonies have smooth surfaces, which act like a mirror in their ability to reflect light; they also tend to have a convex shape, thereby producing a very small image behind the mirror surface at a distance approximately equal to half of the surface radius of curvature. Corkidi et al. explained that when a light source with a small filament is placed in front of the colonies (epi-illumination), a small bright image of the source is produced for each colony. This can be considered analogous to a topographic map if the gray levels are regarded as altitudinal levels. This method allows separation even of overlapping colonies. The method has been validated for a range of contrasting colony types, including *Saccharomyces cerevisiae, Aspergillus nidulans, Escherichia coli, Azotobacter vinelandii, Pseudomonas aeruginos,* and *Rhizobium etli.*

Confluent colonies are separated by other approaches, most notably by establishing colony watersheds (Russ, 1990; Vincent and Soille, 1991), although at low resolution these methods do not adequately resolve confluent colonies (Corkidi et al. 1998). Low resolution in itself will result from counting the whole petri dish with a standard video camera. Mukherjee et al. (1995) used a simple alternative approach, measuring the total area occupied by the colonies and dividing it by the average colony area. This method is also utilized by the IUL Countermat system (Wilson 1995), but it can be clearly established that for a mixed culture with different colony sizes there will be a high degree of inaccuracy. Alternative procedures to resolve confluent colonies are the repeated erosion of the image to a single point per colony. Spreading colonies cause problems for most automated colony counters because they cannot easily be treated differently from non-spreaders. Each colony will therefore tend to be counted as an individual colony.

The current generation of counters tends to use video cameras for data capture, but other approaches have also been used. The laser scanner evaluated by Kramer et al. (1979) used a focused laser beam to detect colonies on an agar plate (see Figure 5.1). Colonies were detected by a reduction in beam energy when a colony obscured transmission to the detector positioned below the plate. Prior to each plate being scanned, a reference transmission level through the agar was initiated and stored by the counter, thus providing self-calibration on a plate by plate basis. When reading spiral plates, the plate is scanned in 500 revolutions of an Archimedes spiral, starting at the outside of the plate. A count would be made only when the edge of the colony is detected for the first time. The drawback of the otherwise excellent counter was an inability to be able to count

on anything but translucent agar. The scanning principle is also used by the CASBA™ 4 counting system (Spiral Biotech), which utilizes a high-resolution CCD (charge-coupled device) line scanner, rather than a video camera, as the reader.

Developments in imaging and computing technology are proceeding at such a rate that it is not possible to review in detail the range of instruments currently available. As instruments are continually being developed, the list of available features continues to grow. The ProtoCOL (Don Whitley Scientific), for example, according to a brochure, "is supplied with comprehensive software facilities that will allow the quantification of every known type of plate. Numerous configurations covering plate type, size limits, count limits, results formats etc. can be adapted, stored and immediately recalled."

Software is available to allow reading of pour plates, spread plates, spiral plates, and even inhibition zones all on one instrument. The current generation of instruments can adequately compensate for different colored agars and for variations in agar depth within a plate. They are able to resolve touching colonies, meniscus effects, different sized colonies, bubbles, sample debris, and even, in some cases, spreading colonies. It is now possible to exclude user-defined areas of the plate and still calculate the mean total plate count.

Attention has also been given to GLP requirements; many instruments will instantly transfer data to secure databases, some of which are also able to hold a reference to the image of the plate. Different levels of user can be controlled by password security systems. If there are occasions where it is necessary to overwrite an automatically generated result with a manual count, the better systems will allow this to happen but will flag the altered result, thereby providing for integrity within the system. Paper copy reports can be generated from most of the available systems.

SOME VENERABLE, INEXPENSIVE, AND LABOR-SAVING METHODS

Several simple, inexpensive labor-saving methods were developed before significant advances in laboratory automation and microelectronics had become commonplace. Some examples are described below.

Loop-Tile Method

The very simple, inexpensive, and underrated loop-tile method can be used successfully with foods or processing environments, and has been recommended, for example, for making total counts in abattoirs and in situations where direct transport of samples to the laboratory is difficult (Kitchell et al. 1973). The apparatus consists of a calibrated platinum loop delivering 0.02 mL drops, a glazed spotting tile, a portable propane or other burner, sterile Pasteur pipettes cut to an external diameter of 1.04 mm, a bottle of sterile diluent, and a set of prepoured agar plates, marked and labeled in quarters. Means for taking samples (e.g., swabs and diluents) must, of course, also be provided.

The technique calls for adding nine drops (0.18 mL) of sterile diluent to each of sufficient tile wells and making successive dilutions through the set by means of the platinum loop, which is flamed at each transfer. Each dilution is plated, in duplicate (but on different plates), by means of the loop, on a marked section of the agar plate. Before the next sample is done, the tile is decontaminated by flaming with alcohol. Agar and incubation times are chosen to suit the sample, but incubation time may sometimes be foreshortened. When counting (preferably under mild magnification, such as a 3-diopter lens), both quarter sections giving a count of at least 20 colonies are used, unless one plate appears to be contaminated. Suspensions for plating should contain at least 2,000 CFU/mL. Spreaders may be a problem, as may microcolonies on plates transported to work sites. In heavily contaminated environments, the diluent bottle and Pasteur pipette should be changed frequently. The efficiency of alcohol flaming for sterilizing tiles should also be checked.

Agar Droplet Technique

This miniature pour plate technique was widely used in Europe for many years. A commercial instrument to assist plating and counting (Droplette) is no longer available; however, even if the technique is performed manually, it can still permit technicians to make 3 times as many total counts per day as by conventional plating (Sharpe and Kilsby 1971). The technique is still finding useful applications (Champagne and Gardner 1990). With some modification it can also be used for counting anaerobic bacteria (Sharpe et al. 1972, 1976).

Agar media (0.5% agar) in 9.9 mL tubes is melted and cooled to 35°C (it remains liquid at this temperature because of the low agar concentration). Dilutions (1:100) of the sample suspension are made in the molten agar by transferring 0.1 mL aliquots with either sterile Pasteur pipettes or drinking straws. Each dilution is plated out, again by straw or Pasteur pipette, as a row of 5 0.1 mL droplets. Because one-hundreth dilutions were made, 3 rows suffice to encompass most contamination levels. Because at least 6 rows of droplets can be placed on a petri dish base and the lid can also be used, 4 or more samples can be plated in one petri dish. Incubation is usually for 24 hours, occasionally longer. A lens should be used for counting, and as many droplets at a suitable dilution are counted to be statistically useful (100–200 colonies); the initial contamination level is calculated by using the appropriate diviser. Because colors in microcolonies are not so easily distinguished as in full-

sized colonies, it is not recommended that specific types of colonies (e.g., red colonies in VRB agar droplets) be counted; the droplet technique is thus a "total count" method, for whatever agar (selective or not) is used. It is suitable for samples containing 1,000 bacteria/g or greater.

Plate Loop

The plate loop count (PLC) was originally described as a smear culture technique (Burri smear) in which a wire loop was used to spread a calibrated volume of milk onto an agar plate surface to enumerate viable bacteria (Burri 1928). The technique has been used by dairy microbiologists for many years; commercial instruments based on it (e.g., Petri-Foss, Foss Electric, Denmark) are especially designed for milk plating. Variations involving the use of oval tubes have been described and evaluated (Myers and Pence 1941; Donnelly et al. 1960). The technique was modified further for use in a pour plate technique (Thompson et al. 1960); reports indicated that it gave results equivalent to the SPC (standard plate count). Tatini et al. (1967) reported that, relative to the SPC, the plate loop technique underestimated the population in raw milk at bacterial densities greater than 10^5/mL; the finding was supported by Wright et al. (1970), who suggested that $SPC=PLC^{1.04}$. An extensive collaborative study of raw milk showed an acceptable reproducibility, based on the Standard Methods criterion (variance <0. 012). The overall log mean by plate loop (1.975) compared favorably with that of the SPC (1.960). However, the overall image of precision and accuracy of the plate loop was significantly different between individual technicians and/or loops. Factors contributing to inaccuracy and imprecision of the plate loop technique included improper loop calibration and significant variation in technique not only among analysts, but also by individual analysts between loops (Brodsky and Ciebin 1980).

METHODS FOR SURFACE CONTAMINATION: AGAR CONTACT METHODS

In several techniques an agar surface is inoculated by pressing it against the test surface. Although the number of organisms transferred to the agar is rarely controllable and does not correlate with methods such as swabbing or the more rigorous blending or stomaching, these contact procedures have the great advantage of providing a "contact print" of contaminants on the test surface. Thus, they can show up localized problem areas that might be missed by more conventional sampling methods —for example, a repeated pattern may indicate inadvertent contact with a part of a machine or deposition of contaminants by a draft.

RODAC Contact Plates

Introduced by Hall and Hartnett (1964) for bacteriological monitoring in hospitals, RODAC™ plates have found widespread use in the food industry and have been widely described and tested (e.g., Favero et al. 1968; Stinson and Tiwari 1978; Terbijhe and Notermans 1981). Their simplicity of use and the print they show of contamination make them eminently suited to the teaching of hygiene practices. Similar contact plates are available commercially from several manufacturers, prefilled or ready to fill. If poured in the laboratory, they should be filled until a convex meniscus is obtained. To use, the agar surface is simply pressed firmly against the test surface with a slight rolling action, without wiping it across the surface. The plate is then incubated for a suitable period.

Agar Syringes and Agar Sausages

The agar sausage technique was first described by ten Cate (1963). It used an agar-filled sausage skin cut to a flat end. After pressing the exposed end against the test surface, a slice of agar carrying the contact print was sliced off with a sterile knife and incubated in a petri dish. Agar sausages were sold by Oxoid under the name "Agaroid" for several years, but they were discontinued because of shelf-life problems. The agar syringe (Walter 1955) is very similar, except that a syringe, from which the end has been removed, is filled with agar. After the sample is taken, the syringe plunger is pushed down a few millimeters so that the exposed agar slice can be cut off and plated. Early devices used glass syringes, but the convenience of plastic syringes was soon discovered. An advantage over the agar sausage is in longer shelf life, because less moisture loss occurs.

Dip Slides and Samplers

Dip slides are useful devices consisting of elastic plastic slides, covered on both sides with sterile agars and enclosed in sterile, plastic tubes. When a slide is dipped into fluids or pressed against surfaces, bacteria adhere to the agar and develop as colonies when the slide is incubated. The slides have been used for the microbiological control of water, milk, foods, and the manufacturing environment (Mara 1972; Genner 1976; Hill 1976, 1977; Griffiths 1978; Mukherji and Simonsen 1981). Dip

slides are also known as AIPC (agar immersion, plating, and contact) slides (Mossel et al. 1976; Mossel and van Rossen 1979), a designation that portrays their versatility more accurately. They are available from several manufacturers, who may custom fill them with specific agar cocktails. A related product, the Millipore sampler (Millipore Corp., United States) consists of a dried, absorbent nutrient layer bonded to a membrane filter. On immersion it absorbs 1.0 mL of fluid. Bacteria develop as colonies on the filter; samplers are available for total, coliform, and *E. coli* counts.

With the exception of using the Millipore sampler, the relation between uptake of bacteria from liquids and their concentration should be determined experimentally. Depending on the nature of the organisms taken up, suspensions containing more than 10^5 CFU/mL are likely to saturate the slides. For slides pressed on surfaces, the limitations and performance of other contact methods can be expected. When testing fluids, a slide should be immersed for several seconds and agitated gently, then removed and allowed to drain (if necessary, the bottom may be touched against a clean tissue), and replaced in its tube, the screw cap should be tightened, and the vial should be incubated for 24 hours at 35°C, or under conditions suitable for the organism of interest. Longer immersion times may be needed for the Coli-Count samplers; for samples that may clog the membrane, up to 5 minutes of immersion should be allowed in order to achieve the proper absorption.

SPIRAL PLATING APPLICATIONS

Spiral platers need not be dedicated to counting applications; the automated sample dispensing and dilution mechanisms have several other applications described below.

MIC Testing

The spiral gradient endpoint (SGE™) test provides for highly sensitive and repeatable MIC (minimum inhibitory concentration) determinations by using the agar dilution technique. One agar plate is equivalent to 8 twofold dilution plates of the conventional agar incorporation technique, thus considerably minimizing the time and materials needed to perform the test. The principle of the test is that an antimicrobial concentration gradient is produced in the agar plate by deposition of a stock antibiotic solution with the spiral plater. Bacterial strains can then be inoculated onto the plate by either swabbing along the radial lines on the surface of the agar plate or by using a Radial Replicator™ (Spiral System Instruments, United States). The MIC can then be calculated from the measured radial location of the transition from growth to no growth. One of the benefits of this technique is that cidal effects that show a sharp transition between growth and no growth can be readily distinguished from static effects, which tend to result in a progressively decreasing colony size in the tail.

The method was first introduced in 1990 (Hill and Schalkowsky 1990; Paton et al. 1990). Wexler et al. (1996) reported on a multilaboratory collaborative study to assess the technique and to compare the data to those obtained by the reference agar dilution method of the National Committee for Clinical Standards (NCCLS) for anaerobic organisms. The standard deviation of the MIC obtained by the SGE test for the 5 participating laboratories was ±0.26 of a twofold dilution, whereas it was ±1 twofold dilution by the reference method. Reproducibility results for two control strains against imipenem, chloramphenicol, and metronidazole indicated that 96% of the measurements fell within ± 1 twofold dilution of the mode value. The equivalence of the SGE test with the NCCLS agar dilution method showed that both were within 1 doubling dilution in 93% of the measurements for anaerobic organisms (n=1,074). Discrepancies were generally with those organism-drug combinations that resulted in trailing endpoints. Similar comparative studies between the techniques have been reported (Hill 1991; Wexler et al. 1991, Hill and Schalkowsky 1990; James 1990; Paton et al. 1990).

Yu et al. (1996) used the spiral plater to quantitatively determine the presence of spontaneous mutational antibiotic resistance among isolates of *Enterobacter* spp. The degree of resistance was estimated by dividing the antimicrobial concentration required to inhibit 90% of the colonies growing in the area beyond the MIC by the MIC itself. When this approach was used, the degree of resistance to third generation cephalosporins and aztreonam was shown to be statistically greater ($P<0.01$) than that to co-trimoxazole, imipenem, and ciprofloxacin. These workers showed the spiral plater to be useful in identifying antimicrobial agents that induce few or no mutants. The advantages of the technique were seen as providing a continuous anti-microbial concentration gradient rather than the conventional twofold dilution.

Mutagenicity Screening

Houk et al. (1991) reported on the spiral *Salmonella* assay as an alternative to the conventional *Salmonella*—mammalicm-microsome mutagenicity assay (Ames et al. 1975; Maron and Ames, 1983). The spiral plater automates mutagenicity testing by serially delivering each component of the assay, bacteria, test compound, and activating S9 mix, one on top of the other. Because the spiral plating delivery system allows fixed as well as variable-dilution depositions along the spiral track, it is possible to present a uniform inoculum of bacteria to a continuous concentration gradient of test compound in the presence or

absence of an S9 mix, all on a single plate. Following incubation revertant colonies can be enumerated. Each spiral plate can therefore provide doseresponse information spanning a concentration range of about 15:1 (Houk et al. 1991).

The advantages of this technique over the standard protocol are a significant reduction in labor, materials, and the need for test compound. Diehl and Fort (1996) validated the spiral *Salmonella* assay against the standard pour-plate assay with 38 compounds from a variety of chemical classes. There was overall test agreement on positive and negative results for 82% of the compounds tested, increasing to 87% when results from only strains TA98 and TA100 were used. It was concluded that the spiral assay was more sensitive in terms of dose response than the pour plate assay.

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6
ATP Bioluminescence and Its Use in Pharmaceutical Microbiology

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ATP (adenosine triphosphate) bioluminescence is a well-established technique for microbial monitoring in the food industry. It has been used extensively for hygiene monitoring and finished product testing for over 10 years. More recently, ATP bioluminescence has gained acceptance within the cosmetic industry and has made inroads in the pharmaceutical industry.

THE SCIENCE

Bioluminescence is an enzymatically catalyzed reaction that generates light. It is seen in nature in glow-worms, fireflies, and certain marine organisms.

ATP bioluminescence used in the laboratory today is based on a reaction that occurs naturally in the North American firefly, *Photinus pyralis.* It is bioluminescence that drives the mate-attracting flash in the female firefly's abdomen. The reaction (see Figure 6.1) was famously first described by McElroy 1947.

Much of what has been published in the scientific literature about ATP bioluminescence need not concern us here. The manufacturers of commercial ATP bioluminescence kits have put all the hard work into requirements such as ensuring that only the D isomer of luciferin is present and that the correct concentration of magnesium is available for the reaction. For an overview of the history and background, see Stanley (1989).

What is perhaps more important to understand is that the ATP bioluminescence reaction is very specific; it can be driven only by ATP and no other molecule. The reaction is also very efficient: almost one photon of light is emitted for every molecule of ATP consumed. This means that there is a linear relationship between the amount of ATP in a sample and the amount of light generated.

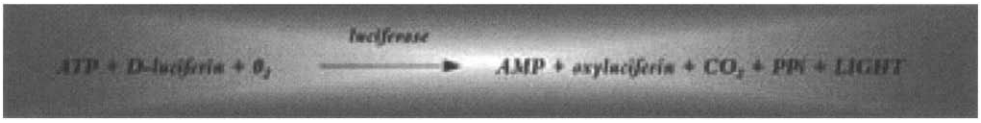
All living cells contain ATP; it is a ubiquitous marker for life. That includes microorganisms, and it is for the detection of bacteria, yeasts, and molds that ATP bioluminescence has been applied most successfully.

Despite the efficiency of the reaction, there is nowhere near enough light generated to be visible by the human eye. Typically, instrumentation is employed to magnify the signal, and results are reported as relative light units (RLU).

The bioluminescence reagents form only part of the test procedure. The ATP must first be released from the microbial cells. Cold detergent extraction has meant that ATP bioluminescence could become an everyday simple test. All of the manufacturers have their own proprietary formulations of extractant.

Several basic protocol formats have been adopted. The simplest of these is in hygiene monitoring (HM). A production surface in the food industry is cleaned and sanitized prior to use. Traditionally, the surface is swabbed and the swab cultured to obtain a microbial count. In ATP bioluminescence HM, it is argued that any result available instantly, and thus allowing immediate equipment for production, must be better than a result obtained several days later. Moreover, the test that is really required is one to demonstrate that the food preparation surface is clean—free from food residues that could act as

Figure 6.1 The ATP Bioluminescence Reaction.



Courtesy of Celsis Ltd.

contamination points or shield microorganisms from the effect of the sanitizer. Therefore, a test that detects ATP, present in food residues, provides not only a more timely result, but also a more appropriate one. This approach has found little application in the pharmaceutical industry, until recently. Hygiena International and Pall have had some success with pharmaceutical hygiene monitoring.

A second basic format requires that a sample from a product normally free from contamination be incubated and the culture tested using ATP bioluminescence. The principle is that if there is a single microorganism present in the sample, it will multiply and become detectable within a shorter incubation time than the wait for colonies that are visible to the eye. Traditionally, many tests on this type of "commercially sterile" product have reported results in terms of counts and have effectively reported zeros more than 95% of the time. This type of protocol is positioned as a screen. A negative result allows the product to be released to market, and a positive result will require the sample to be retested (enumerated and identified) prior to making a decision on whether the product is within specification and can be released. It is this ATP bioluminescence protocol that has found widest acceptance in testing substances ranging from long-life milk and fruit juice to cosmetics and nonsterile pharmaceuticals. This protocol is described in more detail in the section on Celsis RapiScreen™.

This second protocol is modified slightly if the product being tested is very high in natural ATP. A milk or juice sample first receives an ATP-depleting enzyme that acts prior to the microorganisms being extracted. In this way, nonmicrobial ATP is eliminated so that the microbial ATP can be accurately measured.

Attempts to correlate the amount of light with an estimate of microbial numbers have achieved varied success. In its first iteration, this third basic format required that the sample not be incubated; because different microorganisms in different states of stress contain different amounts of ATP, extensive correlation curves need to be generated for each product type. This approach has been useful only in high-bioburden raw products such as meat and raw milk. A more sophisticated approach to the same basic protocol has been adopted by Millipore in the MicroStar ™device (described in detail later), in which the sample is filtered and therefore the microorganisms are spatially separated. This means that during incubation "micro-colonies" develop and can be seen and counted using a CCD (charge-coupled device) camera. This format has been evaluated in the pharmaceutical industry and has gained some acceptance in the beverage industry.

ATP bioluminescence is generally a very robust technique, provided some basic precautions are taken. As ATP is ubiquitous, it is important to ensure that reagents and tips are not contaminated. This is easily achieved by following basic aseptic technique.

It is important to be aware that the sample being tested can interfere with the bioluminescence reaction by either enhancing or quenching the reaction. All of the manufacturers provide basic protocols that allow each product to be tested for these interferences during validation. It is a very simple test, and any interference measured is rarely significant enough to affect the interpretation of a result; even if it is, a simple protocol modification can usually be devised.

THE PRODUCTS AVAILABLE

There are currently three main suppliers of ATP bioluminescence to the pharmaceutical industry. Pall Life Sciences has a system known as the Pallchek™, formerly known as the BioProbe from Hughes-Whitlock; Celsis supplies the RapiScreen system; and Millipore has the MicroStar system. Although there is some commonality among these products, they satisfy different testing requirements; for example, both Pallchek and MicroStar process single samples, whereas the RapiScreen system can run up to 164 samples at a time. As Stewart Green, Wyeth UK, put it, "Potential users must carefully evaluate their individual needs" when choosing a rapid microbiology system.

Pallchek

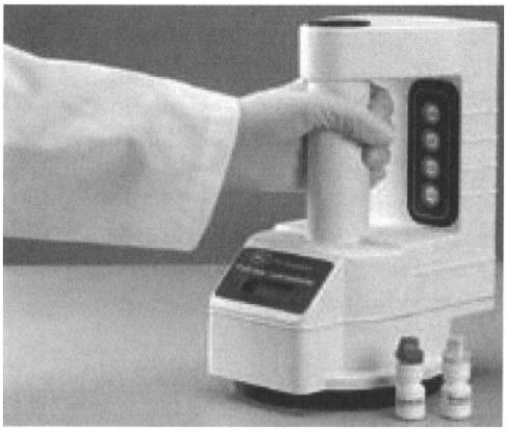
The Pallchek luminometer (see Figure 6.2) is described by the suppliers (Pall Life Sciences, United States) as a highly sensitive, versatile, and easy-to-use system. Although it was originally conceived to be positioned with other hygiene monitoring devices in the food industry, the Pallchek's unique design enables it to meet the needs of a wider audience.

The Pallchek has found a niche in pharmaceutical manufacturing as a quick contamination monitor. The product has a very versatile range of protocols, utilizing membrane filtration, direct measurement on surfaces, conventional hygiene swabs, testing of small volumes of liquid, and filtered incubated samples. The instrument is small and portable and is not expensive. The Pallchek tests one sample at a time, each individual measurement taking less than one minute.

Different reagent kits are available that provide the user with different levels of sensitivity. There are two High Sensitivity Kits, one of which contains an "Activator," claimed to improve detection where high levels of injured cells are suspected. In the most sensitive procedure, utilizing membrane filtration, the Pallchek (BioProbe) can detect as few as 10–100 cells/100mls of filtered sample.

As recorded by Newby (2000), the potential uses for the Pallchek system in the pharmaceutical industry could include water testing, noncritical environmental monitoring, in-process bioburden, and pre-filtration bioburden testing. Newby and Johnson

Figure 6.2 The Pallchek (BioProbe) Luminometer.



Courtesy of Pall Life Sciences

(Chapter 4) regard the Pallchek as having potential within the pharmaceutical industry as a screening device to indicate microbial levels in various aspects of pharmaceutical manufacturing. Others see a potential for the Pallchek to be used for monitoring of purified water (Cundell 2000). There are five different ways of using the Pallchek:

1. To detect organisms on filters
   The sample is filtered and the membrane placed on the test plate. Reagents are added and the Pallchek applied. This procedure allows the working volume to be increased and therefore the sensitivity of the test to be increased. In addition, testing of a larger sample volume is statistically more significant.
2. To perform direct measurements on work surfaces
   Surfaces can be measured directly by adding reagents and applying the Pallchek. Unlike most instruments, the Pallchek does not require samples to be inserted into cuvettes for analysis. Reagents can be placed directly on a surface and the Pallchek placed directly on top to perform the measurement. The company claims that this eliminates many of the errors associated with swabbing. (Whitlock 1994).
3. To analyze conventional hygiene swabs
   To assess the hygiene of areas that are difficult to access, such as plumbing, a standard hygiene swab is used. The swab is placed on a disposable sample holder already positioned on the test plate, reagents are added, and the Pallchek is applied.
4. To detect organisms directly in liquids
   Direct analysis of small volumes of liquid samples, typically 0.1 ml, can be achieved by pipetting the sample directly onto a disposable sample holder already positioned on the test plate and adding reagents prior to applying the Pallchek.
5. To detect presence or absence of organisms in filtered samples
   Direct evaluation of incubated samples can be carried out by placing the membrane filter on plated selective agar and incubating for 24 hours. The plate is then placed directly on the test plate, reagents are added, and a measurement is taken.

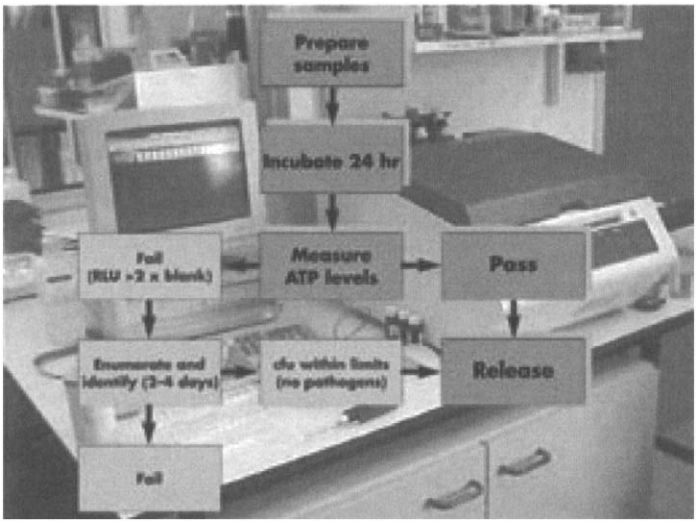
The Pallchek instrument stores up to 500 sets of data within the luminometer. Data stored in the instrument can be downloaded to an Excel spreadsheet for further manipulation or analysis. Data can also be printed by attaching a small printer to the luminometer directly.

RapiScreen

The Celsis RapiScreen system is one of the simplest rapid methods available. It is based on the fact that the vast majority of nonsterile products manufactured today are free of any bioburden. In practice greater than 95% of samples tested give a result of zero. Therefore, the reasoning is, why go to the trouble and expense of doing a total aerobic count and a yeast and mold count and demonstrating the absence of four or more pathogens when the chances are there is nothing there? This screening process is shown in Figure 6.3.

By carrying out enrichment and testing using ATP bioluminescence, a pass or fail result can be obtained in 24 to 48 hours, and the product can be released to market. In the small proportion of samples for which the RapiScreen result is a fail, the product can be retested by the conventional method to allow enumeration and identification. It is possible, or course, that some of the products retested can be released anyway because they have an acceptable bioburden; at worst, this minority has

Figure 6.3 The screening prinicple.



Courtesy of Celsis Ltd.

been delayed by just one day. There are very real savings to be had, and Celsis has pioneered a financial model to demonstrate this.

The RapiScreen test works as follows. The sample preparation is as similar to the current method as possible; the following is given as an example. A 10 g sample of product is suspended in 90 ml of broth (see Figure 6.4) in a sterile container.

The product in broth suspension is incubated at 30–32°C, being shaken at 250 rpm (see Figure 6.5). After 24 to 48 hours incubation, 50 µl of the enrichment culture is added to a cuvette and placed in the luminometer. The luminometer automatically injects 200 µl of LuminEX, a reagent that extracts the ATP from the microbial cells. Next, 100 µl of the LuminATE (bioluminescence reagent) is injected, and the light is measured in RLU. The result can be printed or downloaded to a computer through the Celsis proprietary software Advance.im.

The broths that Celsis recommends are Difco Letheen or Difco TAT. Difco is the preferred supplier, as Celsis has shown Difco broths to be consistently and reliably low in ATP. Elevated levels of ATP in the broth will reduce the sensitivity of the overall assay. The pharmacopoeial broth is Casein Azolecithin Tween, which both Letheen and TAT are based on. The TAT formulation contains a higher concentration of the neutralizing agents and is therefore preferred in some circumstances; the presence of tricolsan in products is an example quoted.

It is perhaps surprising that Celsis has chosen the incubation conditions quoted. Conventionally, microbiologists have been taught that yeasts and molds grow better at a lower temperature than bacteria. This is untrue, as was demonstrated independently by Marshall et al. (1998). The practice of using the low incubation temperature traditionally applied to yeast and mold incubation was for purposes of identification. At around 25°C, yeast and mold grow in a perfect morphology that allows them to be identified optically. For the purposes of generating bioburden as rapidly as possible to demonstrate the presence or absence of these organisms, 30–32°C has been shown to be the best compromise temperature for the broadest range of organisms.

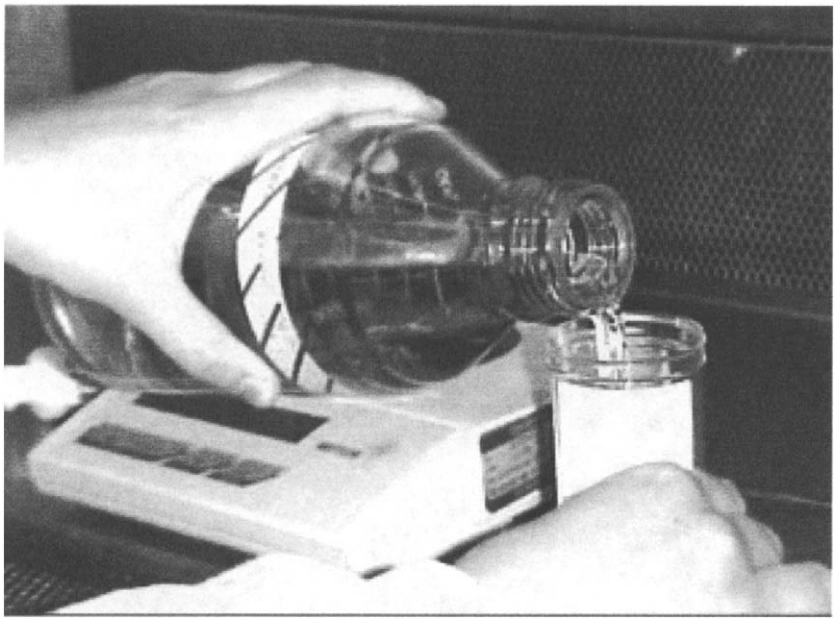
Shaking too is not used traditionally in quality control microbiology, but it is commonplace in the biotechnology industry. Where the generation of large amounts of biomass is the business, agitation to ensure complete aeration and well-mixed nutrients is nothing new. Celsis has demonstrated that, for some organisms at least, shaking can make the difference between the organisms growing to detectable levels in the time allowed or being missed.

The Celsis RapiScreen system is currently able to run on one of two “sister” luminometers. The Celsis Advance is capable of running up to 150 samples at a time, taking about an hour to process them (see Figure 6.6). The coupe is a smaller version, able to run only 30 samples, but it may meet the needs of some laboratories better (see Figure 6.7). It is also equipped with an on-board printer.

The Advance.im software is an optional extra that allows users to operate the luminometer and manage the results through the graphic user interface (see Figure 6.8). Celsis claims that the software has the appropriate security and audit trails to ensure compliance with 21 CFR Part 11.

The RapiScreen system is perhaps the most widely used of the ATP methods. Satisfied customers who have published their experiences include SmithKline Beecham (Kay 1999), Colgate Palmolive (Preston 2001), and Wyeth (Green 1999). These, along with Abbott, have also presented papers on the Celsis system at international meetings. Many other users have been unable to publish their experiences because of company confidentiality restrictions.

Figure 6.4 Sample preparation.



Courtesy of Celsis Ltd.

Figure 6.5 Sample incubation.

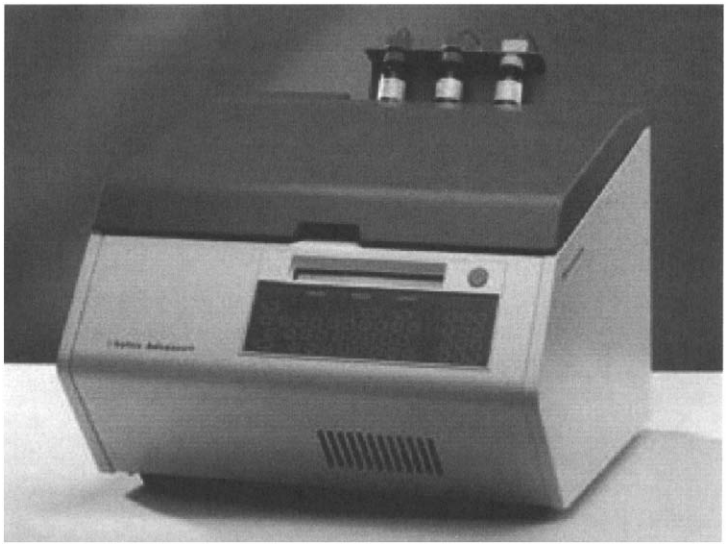
Courtesy of Celsis Ltd.

Celsis RapiScreen was the first rapid method for finished product release that has been approved in Product Marketing Authorizations, and it remains the only one. Wyeth achieved this approval by applying to the UK Medicine Control Agency (MCA) for a Type 1 Amendment with such approvals to the product marketing authorization for a test product. It was approved without question, on the basis that should a "positive" result be achieved, it would be confirmed by traditional methods. The regulatory agencies have now approved Type 1 Amendments to Product Marketing Authorisations to include RapiScreen, from several different companies, for products sold across the world, from Australia to Yemen. Further, a new Community Marketing Authorisation has been approved by the European Medicines Evaluation Agency (EMEA), which included RapiScreen as the microbiology release test.

MicroStar

The Millipore MicroStar system (see Figure 6.9) is a unique combination of three technologies that enable the enumeration of microorganisms in samples. The first is membrane filtration, through a specialized hydrophobically partitioned membrane,

Figure 6.6 The Celsis Advance™ luminometer.



Courtesy of Celsis Ltd.

Figure 6.7 The Celsis Advance™ coupe luminometer.

Courtesy of Celsis Ltd.

designed to optimize the enumeration of microorganisms. The second is ATP bioluminescence. The third is intensification of the bioluminescence from each cell (or microcolony) thousands of times and capture of the light signals with a CCD camera (see Figure 6.10). An image processor enumerates the microorganisms and displays them on screen.

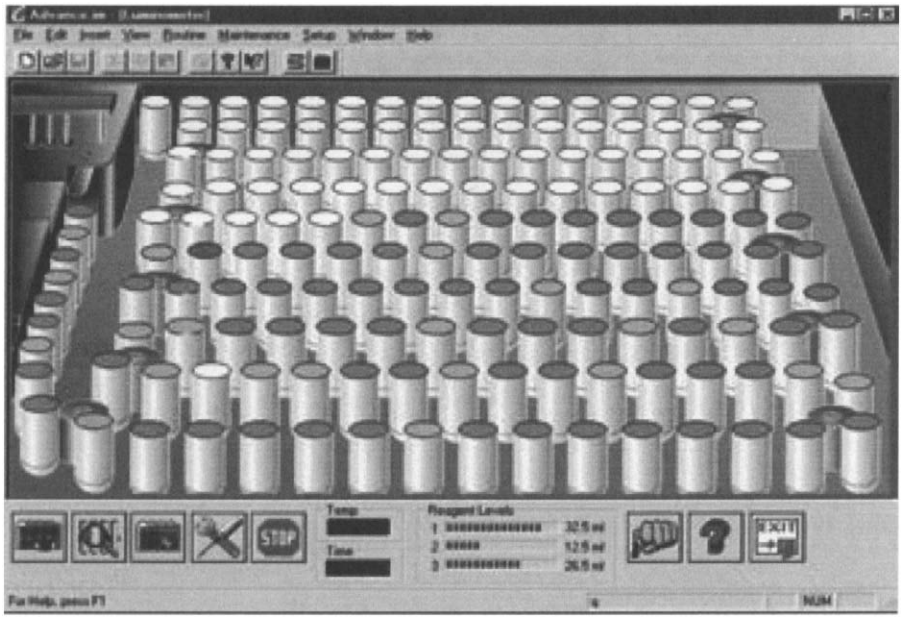
The MicroStar test works as follows. The sample (<1 ml to 1 L, or more) is filtered through presterilized, disposable Milliflex™ filter units, with the unique MicroStar membrane (see Figure 6.11). Growth inhibitors are rinsed away by filtering with an appropriate rinse solution.

The filter base is then released and the filter unit transferred to the AutoSpray station for application of the ATP releasing agent (see Figure 6.12). For yeast or vegetative mold detection, reagents are applied immediately, but for bacteria detection, incubation is required. The AutoSpray station is preset to apply the appropriate spray volume evenly across the membrane. The spray step is repeated to apply the ATP bioluminescence reagent.

Finally, the filter unit is transferred to the MicroStar detection tower for counting (see Figure 6.13). Information about the sample is entered into the computer, and a key press initiates the count.

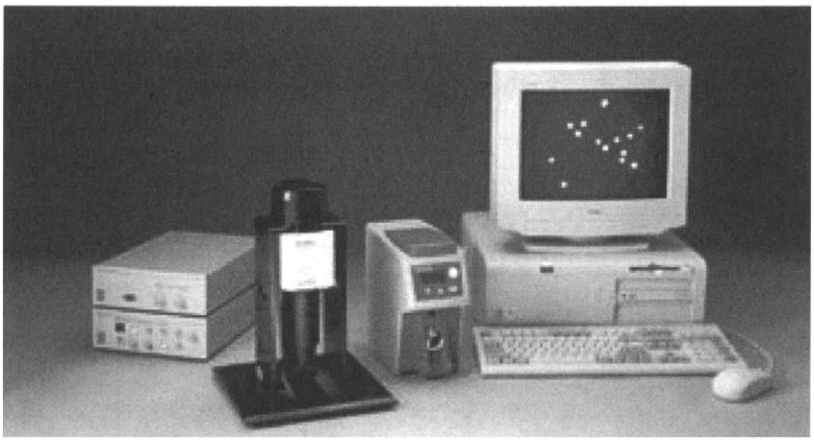
The count and an image of the filter are automatically recorded, displayed, and archived (see Figure 6.14).

Figure 6.8 The Celsis.im software.



Courtesy of Celsis Ltd.

Figure 6.9 The Millipore MicroStar™ system.



Courtesy of Millipore, Inc.

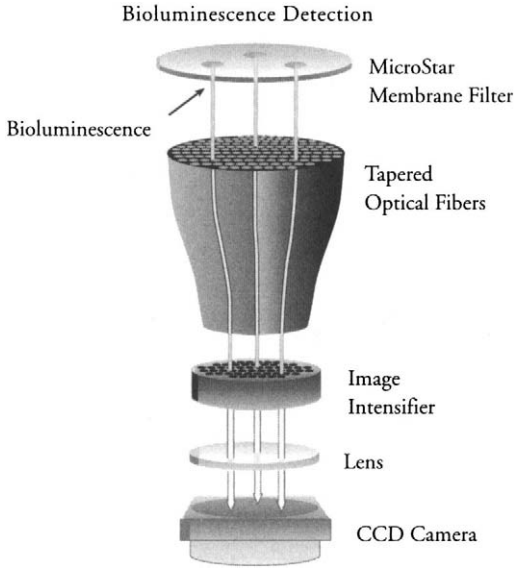
Applications include water, raw materials, in-process bulk solutions, CIP (clean-in-place) testing, environmental testing, final product testing, and sterility screening

Millipore claims that the MicroStar system is able to detect yeast cells immediately, because of the high ATP content, and to detect bacterial cells in one-quarter of the time taken by conventional methods. MicroStar provides results in colony-forming units directly, without the need for calibration curves, which may be more acceptable to some users.

THE FUTURE

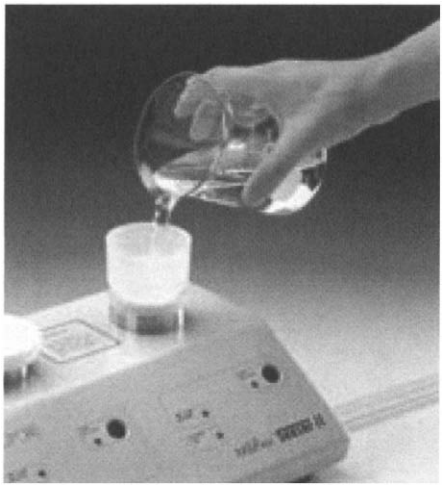
Despite the success of some of the systems described in this chapter, the drive within pharmaceutical microbiology is for an ever more sensitive and therefore faster test. Single-cell detection in real time and, ideally, knowing what that cell is would be the ultimate goals. ATP detection is not there yet. Methods are being developed to amplify the amount of ATP available in order to improve the sensitivity of the test. Celsis has launched the AKuScreen system in the personal care market. This product uses the adenylate kinase reaction; that is, it drives the intracellular enzyme that makes ATP to produce 1,000 times more ATP by the addition of ADP (adenosine diphosphate). This technology has yet to be proven and validated in the pharmaceutical arena, but it does have potential to improve the sensitivity and reduce the time to detection of ATP bioluminescence.

Figure 6.10 The CCD camera.



Courtesy of Millipore, Inc.

Figure 6.11 Filtering sample.



Courtesy of Millipore, Inc.

SUMMARY

Although pharmaceutical microbiologists have been slow to adopt new methods, ATP bioluminescence is gaining credence as a valuable technique. It is the only rapid microbiological method that has, to date, been accepted by regulatory authorities. As with all techniques, there are limitations to ATP bioluminescence, but pharmaceutical microbiologists do have this viable alternative to plates in the twenty-first century.

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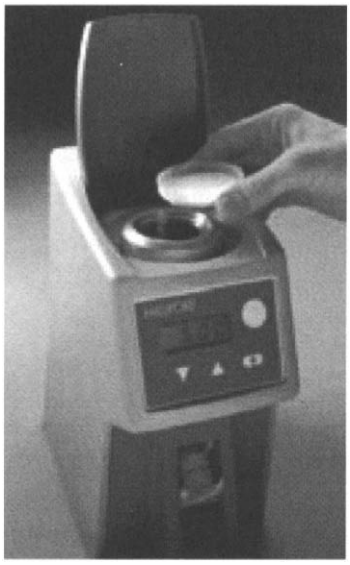
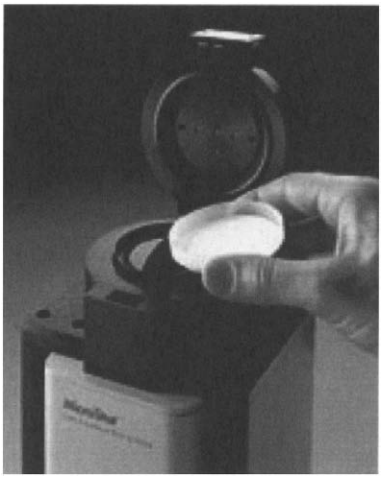
Figure 6.12 Placing the membrane in the AutoSpray station.



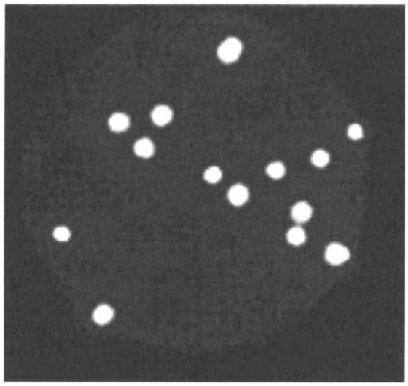
Courtesy of Millipore, Inc.

Figure 6.13 Transfer of the filter to the MicroStar detection tower.



Courtesy of Millipore, Inc.

Figure 6.14 An image of the filter as recorded by the MicroStar.



Courtesy of Millipore, Inc.

7
Impedance

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Interest in the field of rapid and automated methods in the microbiology laboratory has grown steadily over the past 20 years, although few of the methods currently available are recognized as standards. There are exceptions to this rule, and in the next few years the number of exceptions will grow. One of the limiting factors to their acceptance has been the ongoing requirement for third-party laboratory accreditation and the use of validated methods. Consequently it is often easier for a laboratory to include established standard methods rather than new and often unvalidated methods. Despite this there is a driving force in the industry to adopt new microbiological methods, the main drivers being increased method sensitivity, faster results, and the need to reduce labor costs in the laboratory. Impedance microbiology can address all these issues but has tended to focus on producing results more quickly than is achievable by conventional methodology and making a significant contribution in reducing labor costs through automation. It is interesting to note that even given the relatively widespread use of impedance techniques since the late 1970s, Kell and Davey (1990) concluded that the generalized impedance approach remains astonishingly underexploited.

If indeed impedance offers advantages of quicker results, then it is useful to be able to quantify this parameter. Eden and Eden (1984) introduced the term "rapidity ratio," which, although not widely used, does merit further consideration as a means for comparing technologies.

Rapidity ratio was defined as the ratio of time t_p required by the standard method over impedance detection time t_p for the same sample. The time required for detection by the standard plate count method will usually be 24 or 48 hours. The ratio will, of course, increase for higher bacterial concentrations.

The advent of electrical detection systems has in part been a result of advances in computing and the ability to multitask and to handle copious amounts of data. Electrical measurements are instant, and after initial setup the tests require no further reagents and no operator involvement. The systems can be preprogrammed to sample every 5 minutes, thereby increasing sensitivity and outputting positive results on the screen as they occur. Rather than having to wait for a predetermined incubation period of 1 to 4 days before reading, the researcher can obtain results in real time. Electrical systems can respond at best to a threshold microbial concentration of 10^3/mL, although more usually it will be in the range 10^5 to 10^6/mL. An inoculum of one organism will be detected when it reaches this threshold; this is dependent on all the usual growth parameters as well as the electrochemical potential of the medium.

Measurement of electrical impedance has considerable versatility and widespread potential to offer the pharmaceutical industry. Electrical impedance is a measurement of microbial metabolism and growth, and this measurement can be continuous. Thus, there are strong similarities between impedance and the conventional cultural methods that may make it easier to satisfy compendial and regulatory requirements of validation and equivalence.

HISTORY OF DEVELOPMENT

Impedance microbiology originated at a meeting of the British Medical Association in Edinburgh in July 1898 where G.N.Stewart presented electrical response curves from the putrefaction of defibrinated blood (Stewart 1899). Although the form of the curves was very similar to those obtained from available instrumentation, it was clear that Stewart was not measuring in the same way, because his experiments lasted more than 25 days. The measuring system he used was two balanced platinum electrode cells, and the temperature was kept constant under running water. The work of Stewart was followed by that of Oker-Blom (1912), who commented in his paper that the technique had not been widely accepted as one of the major methods of bacteriological measurement. It wasn't until the mid-1970s that the technique began to receive the

attention it merited, coinciding with the introduction of commercially available instruments. Further publications, notably by Parsons and Sturges (1926) and Parsons et al. (1929), who worked with *Clostridium* spp., demonstrated the ability to relate conductivity change to ammonia production. In these studies too, electrical changes were measured over 10 to 20 days. Within 10 years Allison et al. (1938) had further extended this work and shown good correlation between conductivity reading and changes in pH, carbon dioxide, ammonia production, and numbers of organisms. It wasn't until McPhillips and Snow (1958) electrically monitored acid production in milk by *Streptococcus lactis* that we were to see curves similar to those from current instrumentation.

The introduction of dedicated impedance systems gave rise to an increase in published literature, beginning with the notable work of Ur and Brown (1973, 1974, 1975), Cady (1975), and the particularly important work at the Torry Research Station (Richards et al. 1978). The influential book *Impedance Microbiology* (Eden and Eden 1984) became a reference text for all impedance microbiologists; it is now out of print, but not out of use.

IMPEDANCE THEORY

Impedance can be simply defined as the resistance to flow of an alternating current as it passes through a conducting material. For a detailed review of impedance theory, see the publications by Eden and Eden (1984), Kell and Davey (1990), and Blake-Coleman (1993). When an electrolyte solution is exposed to an electrical field, the cations will move toward the negative cathode and the anions toward the positive anode. The movement of ions constitutes current flow within the solution, each ion carrying a fraction of the current in proportion to its degree of mobility and concentration. Conversely capacitance stores energy in an electrical field; a typical capacitor will consist of two conducting surfaces separated by a dielectric material. If a DC voltage is applied to a capacitor, current flow is prevented, but an AC voltage will produce current flow proportional to the rate of voltage change.

It is sufficient for our purposes to consider, as first proposed by Warburg (1899, 1901), that when two metal electrodes are immersed in a conductive medium the test system behaves either as a resistor and capacitor in series or as a conductor and capacitor in parallel. In the first case, the impedance of the system is measured as the dependence of the voltage on the current, the system being considered connected to a current of infinite resistance. In the second case, the system is considered connected to a voltage source of zero resistance, and the admittance describes the dependence of the current upon the voltage (Kell and Davey 1990)

In the case where the system is treated as a series combination, application of an alternating sinusoidal potential will produce a current that is dependent on the impedance *(Z)* of the system, which in turn is a function of its resistance *(R),* capacitance *(C),* and applied frequency (F):

$$z = \sqrt{R^2 + (\tfrac{1}{2}\ \pi\ \mathrm{FC})^2}$$

Any increase in conductance, defined as the reciprocal of resistance, results in a decrease of impedance and an increase in current. The AC equivalent of conductance is admittance, defined as the reciprocal of the impedance. The units of impedance measurement are Siemens (S). Microbial metabolism usually results in an increase in conductance and capacitance; thus as microorganisms grow the impedance signal actually decreases. It therefore is preferable to follow the conductance and admittance signals, thereby positively reflecting the increase in metabolic activity arising from microbial growth.

It is clear that impedance is a complex term that comprises capacitative and conductive components, both of which are dependent on the frequency of the alternating current applied to the electrode system. At low frequencies the impedance reading is largely affected by the capacitance component, whereas at high frequencies it is predominantly affected by conductance.

Direct Impedance

It can be readily appreciated that changes in impedance of the growth medium result directly from the changes taking place in the bulk electrolyte. Substrates in microbiological growth media are generally uncharged or weakly charged but are transformed into highly charged end products as organisms follow normal metabolic pathways, thus increasing the conductivity of the test medium. Simple examples include the conversion of glucose from a non-ionized substrate to two molecules of lactic acid and a corresponding increase in conductivity. Further metabolism will take the lactic acid and three oxygen molecules to carbonic acid; the resulting three ion pairs include the smaller, more mobile bicarbonate ion, which is a more effective electrical conductor than the lactate ion. Hydrogen ions are nearly seven times more effective conductors than sodium ions (Eden and Eden 1984); therefore, one might predict that a weakly buffered medium would allow a greater impedance change than a more strongly buffered medium. For a more detailed appraisal of the effect of medium buffers on conductance, see the work of Owens (1985). It is important to stress that the principles of medium design, fundamental to traditional microbiology, are equally if not more important in impedance microbiology. First, a medium must be chosen that

will support and select for the growth of the test organism. Second, that medium needs to be optimized for an electrical signal. This is well illustrated by *Staphylococcus aureus,* which will grow in nutrient broth but does not produce a significant electrical response, whereas in Whitley Impedance Broth® (Don Whitley Scientific Ltd., United Kingdom), not only does it grow well but it also produces a strong impedance signal. The growth of some organisms, particularly yeasts and molds, does not result in large changes in impedance. This is considered to be due in part to the fact that they do not produce strongly ionized metabolites, but rather non-ionized end products such as ethanol. Moreover, Suomalainen and Oura (1971) have shown that yeasts can absorb ions from solution, resulting in a net decrease in medium conductivity.

An impedance system can therefore be considered simply as measuring net changes in impedance in the culture medium at regular intervals. When a test is initially set up, the user defines the test criteria; when the rate of change of impedance exceeds this predetermined value, the system will detect growth. The time to detection (TTD) is a function of the size of the initial microbial population, the growth kinetics of the test organism, and the properties of the test medium. For a given test protocol, the TTD is inversely proportional to the initial microbial loading of the sample. At the point of detection, it is generally considered that there will be approximately 10^5 to 10^6 CFU/mL of the test organism present in the system. This will vary depending on organism type and medium, but will be constant for any organism growing under defined test conditions. It is important to differentiate between this detection threshold and the sensitivity of an impedance system capable of detecting the presence of organisms at levels as low as <10 CFU/mL, providing the organisms are viable. It is well established that the electrode construction, stainless steel compared to platinum, will affect sensitivity of the test system. Eden and Eden (1984) showed that electrodes located at the bottom of a test cell resulted in detection thresholds 1 log cycle lower than with the same electrodes located at the top of the test cell.

The fact that real-time microbial activity is being measured rather than the activity at a single point in time is a significant and powerful feature, one that enables the system to detect the presence of low numbers of organisms. Several factors will affect time to detection. TTD will correlate only with the initial concentration of test organisms, providing that the generation time of the test population is more or less constant under the experimental conditions. Therefore, not only does incubation temperature need to be kept constant because of physico-electrical properties, as discussed earlier, but also because it will have a direct effect on the generation time of microorganisms.

Indirect Impedance

High salt concentrations are routinely used in many selective media and may be present in some test samples. For example, LiCI is incorporated into Baird-Parker staphylococcal medium at 5 g/L and into Oxford *Listeria* medium at 15 g/L. Also $MgCl_2$ (36 g/L) is used in Rappaport-Vassiliadis broth for *Salmonella* isolation. The resultant high-impedance readings of these media are outside the normal working range of the direct impedance technique. However, using the indirect technique, the researcher can overcome these problems by monitoring micro bial metabolism via the production of carbon dioxide. In this instance potassium hydroxide is added to the impedance cell to bridge the electrodes. The inoculated culture medium is in a separate chamber and not in contact with the electrodes or potassium hydroxide. The unit is tightly sealed, so that any carbon dioxide produced as a result of normal metabolism is absorbed by the potassium hydroxide, causing a resultant decrease in impedance.

Principle of Detection by Indirect Impedance

The detection of carbon dioxide by indirect impedance was first described by Owens et al. (1989). Carbon dioxide produced as a consequence of microbial metabolism will dissolve in the aqueous growth medium until the solubility product is exceeded and the volatile gas diffuses into the headspace. In routine use the electrodes of the test measuring cell are bridged by a potassium or sodium hydroxide plug containing agar; alternatively, a simple solution may be used. As the CO_2 is evolved, it reacts with the alkaline solution, forming a carbonate with a reduced conductivity reading. This can be explained by the following reaction:

$$CO_2 + 2OH^- \rightarrow CO_3^{2-} + H_2O$$

The pH of the test system is important, because the amount of CO_2 in solution is governed by the Henderson-Hasselbach equation that relates to the ionization constant of the carbonate formed in the culture, the pH, and the relative amount of volatile CO_2 available.

A key factor in the design of the systems is that the measuring electrodes are separated from that part of the cell containing the growth medium. The indirect impedance method has several advantages relative to the direct conductance method. Indirect impedance allows the use of conventional media formulations, which thus need not be optimized to produce an optimal impedance response. Media containing high salt concentrations that are outside the measuring range of the direct method can be used. It also allows detection of some microorganisms that produce small or no detectable impedance changes by the direct

method. Indirect impedance can also be used with sample types that may physically interfere with the electrodes in the direct method.

The dynamics of carbon dioxide absorption and the ratio between the impedance variation and the amount of carbon dioxide produced have been investigated (Dezenclos et al. 1994). After carbon dioxide was injected either directly into the potassium hydroxide solution or above it, optimal results were obtained with potassium hydroxide (5–6 g/L) in a volume of 0. 7–1.2 mL, Impedance changes of 280 µS/µmol carbon dioxide were obtained at 27°C with potassium hydroxide concentrations of 0.5–8 g/L. This agrees well with the result predicted by Owens (−278.6 $S.cm^2.mol^{-1}$ carbon dioxide absorbed; Owens et al. 1989). Not surprisingly the results were temperature dependent. While Owens et al. used aqueous solutions of 0.03 and 0.04 M KOH, Bolton (1990) used a semisolid 0.04 M KOH, and Druggan et al. (1993) used a 0.06 M semisolid KOH preparation. It is advisable that these alkaline solutions be degassed before use.

The work of Bolton (1990) has shown the indirect technique to be a powerful tool for working with strains of *S. aureus, Listeria monocytogenes, Enterococcus faecalis, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Aeromonas hydrophila,* and *Salmonella* spp. With this method, the medium does not necessarily need to be optimized for electrical response, allowing media previously considered unsuitable to be used for impedance applications. Applications of indirect impedance technology also have considerable potential for workers who require a rapid, easily manageable, highly sensitive system for monitoring and quantifying CO_2 production, whether in whole cell or isolated enzyme studies.

INFLUENCE OF ELECTRODES

Although most attention has been given to the effects of microbial growth on the changes in conductance of the culture medium, it is important to acknowledge the effect on capacitance of polarization of the electrodes. Ions close to the electrode do not undergo the same forces as those in the bulk solution, resulting in a different electrical field structure around the electrode compared to that in the bulk solution. This process leads to what is termed the interface region, which is a compromise between the phases. The interface region is electrically neutral, with a potential difference across the interface. The arrangement of charges and oriented dipoles in this region is referred to as the electrical double layer (Eden and Eden 1984).

Richards et al. (1978) showed the capacitance of the electrode polarization to be relatively insensitive to *E. coli* metabolism (although this work was carried out with platinum electrodes). Hause et al. (1981) showed that irrespective of bacterial species, electrode material, or configuration, there was an increased impedance signal at low AC frequencies, in the range 100 to 1,000 Hz, whereas at 10,000 Hz the signal was almost entirely conductance. The changes in capacitance component were always greater than for the conductance signal. Electrode structure and preparation are reviewed thoroughly by Blake-Coleman (1993). It is important to point out that although electrode configuration does not appreciably affect detection time, it can have a considerable effect on the magnitude of the total electrical change detected by the respective instrumentation.

Traditionally two-electrode systems have been used in commercial instrumentation; however, the introduction of the BacTrac® 4000 (Sy-Lab, Austria) has given rise to a four-electrode configuration.

PRINCIPLE FACTORS AFFECTING DETECTION TIME

It has been established that time to detection (TTD) is inversely proportional to the numbers of organisms present in the initial test sample, and so the first factor affecting TTD is concentration of test organisms (see Figure 7.1). TTD will correlate with the initial concentration only if the generation time of the test population is kept constant under the described experimental conditions. This is of particular importance when dealing with antimicrobial compounds.

Concentration of the test growth medium will affect the impedance signal. Dilute media have a low ionic strength, and so a given change in conductance arising from microbial metabolism will be relatively large. Additionally, low concentrations of growth media may not fully support the nutritional needs of the test organisms; the results can be slower growth.

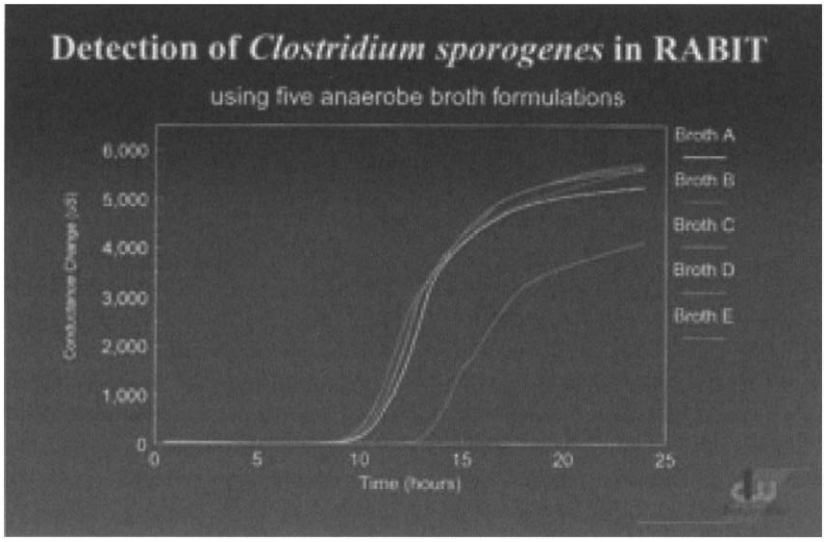
Temperature is also critical; it will affect generation times and also both capacitance and conductance components of the impedance signal. Eden and Eden (1984) showed that a temperature increase of 1°C will result in an average increase of 0.9% in capacitance and 1.8% in conductance. It thus becomes evident that if temperature is not kept constant, the electrical signal might simply be reflecting temperature change rather than microbial growth.

INSTRUMENTS

At present four manufacturers are producing impedance instrumentation:

Bactometer®	bioMérieux UK Ltd.

Figure 7.1 An example of results generated by impedance detection (RABIT).



BacTrac®	Sy-Lab
Malthus®	Malthus Instruments Ltd.
RABIT ®	Don Whitley Scientific Ltd.

All the systems are automated and can produce detail reports.

Bactometer

The Bactometer system followed from the work of Cady (1975) and employs the principle first used by Stewart (1899) in having paired and electrically balanced test cells, one of which acts as the control and the other as a test cell. The test cells are disposable, have stainless steel electrodes, and are arranged in modules of 16. Cells are incubated in an air incubator, where the temperature is controlled in the range ambient to 55°C. Readings are taken over time and the "out of balance" signal calculated. One of the Bactometer's distinguishing features is that it operates in a choice of modes—conductance, impedance, and capacitance. Although any combination can be selected, obviously only one mode can be used at any one time. The Bactometer signal is characterized as a ratio and thus is dimensionless.

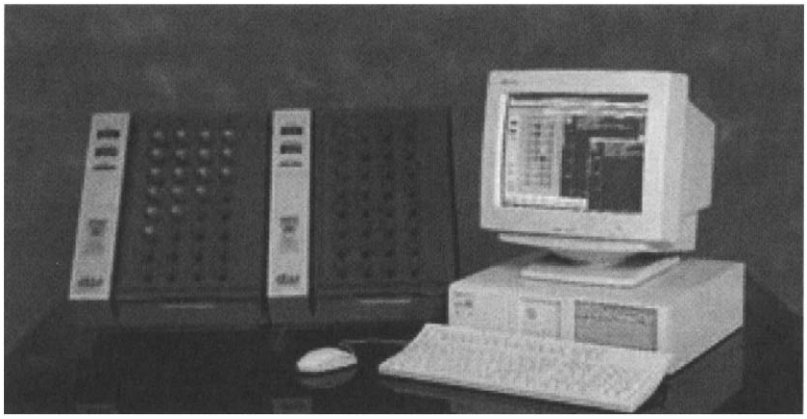
BacTrac

The BacTrac has a different type of electrode arrangement. It employs four electrodes, one pair for measuring solution changes and the other pair for electrode changes. The detection system, which has been referred to as the impedance-splitting method, simultaneously evaluates two electrical parameters during growth of microorganisms: the change of the media impedance, Z_M, referred to as the M-value, and the change of the so-called electrode impedance, Z_E, referred to as the E-value. The BacTrac system also displays relative values. The M-value represents the growth mediums impedance change with time and is expressed as the percentage decrease of the initial value; The E-value represents the same for the electrode impedance change. Percentage values offer the advantage of identical starting points (0%), allowing easy comparison of various graphs; however, they do not reflect absolute values, which can be important in comparative studies.

Separate Registration of M-Value and E-value

The BacTrac measuring system allows for the investigation of microorganisms by using two simultaneously registered electrical curves. Whether both or just one is used for microbial determination depends on the application. Generally, the E-value is considered the more sensitive quantity, showing an earlier reaction, However, because of this high sensitivity, this quantity is more prone to scattering. In cases where many ions are a priori present in a suspension, the suspension features a high conductivity. Measurements of conductivity, conductance, or resistance (impedance, M-value) thus do not show any changes over time; these situations then favor the use of the E-value. BacTrac offers reusable and disposable cells.

Figure 7.2a The RABIT impedance system.



Malthus

The Malthus instruments, based on the work of Richards et al. (1978), were initially developed at the Torry Research Station in Aberdeen, Scotland. The Malthus instrument offers a variety of test cell configurations, each of which carries a pair of inert electrodes. In the original instruments, these were made of platinum bonded to a ceramic carrier, constituting thick-film technology. These electrodes could be reused and could withstand autoclaving. Malthus Instruments Ltd. now offers single-use disposable electrode cell systems with titanium electrodes. Test cells containing media and inoculum are incubated in water incubators whose temperature is controlled to ±0.005°C in the range 5–55°C. The instrument essentially monitors resistance on each cell at predetermined time intervals of 6–60 minutes, The software converts the data to conductance, which is displayed on screen.

RABIT

The RABIT (Rapid Automated Bacterial Impedance Technique) is a modular system capable of monitoring up to 512 individual tests, 32 tests being accommodated in a single incubation module and each module capable of being run at a separate temperature (see Figures 7.2a and 7.2b). The solid aluminium heating block allows for precise temperature control. The test cells are fully autoclavable and extremely robust, because of their special-grade stainless steel electrode. Indirect and direct impedance techniques can be used in the same cell; each cell has a working volume of 2 to 10 mL. The user has complete access to all test cells throughout the incubation period, and individual cells can be initiated or terminated at any time without affecting other tests. A Statistical Process Control (SPC) interface is available allowing for results to be directly downloaded into the SPC software. RABIT test cells (see Figure 7.3) also are available to allow for in-use observation of the system.

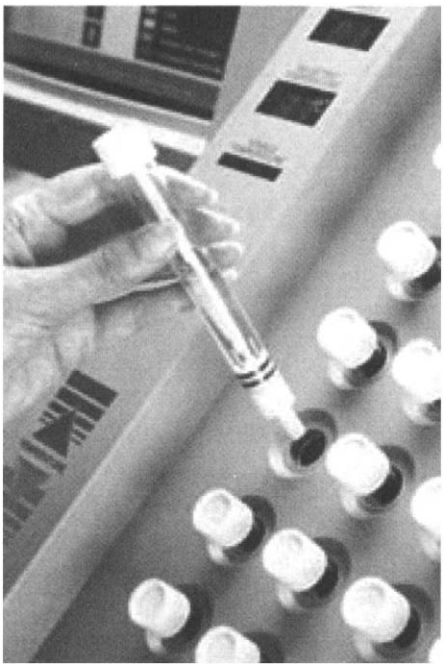
PHARMACEUTICAL APPLICATIONS OF IMPEDANCE

Throughout all stages of the development and manufacture of pharmaceutical products, the determination of microbial counts and the detection and identification of pathogenic organisms are an integral part of the preparation of products that satisfy the regulatory authorities. Reports in the literature suggest that impedance could have many potential uses for routine microbiology in the pharmaceutical industry. Impedance technology has so far been applied mostly to the food industry, but many of the now established methods can be shown to be relevant to the pharmaceutical industry also.

Enumeration of Bacteria and Fungi

Most of the tests used to ensure the quality of a pharmaceutical product are currently dependent on determining the total viable count of microorganisms. The test for microbial contamination, for example, provides limits for the number of bacteria and fungi allowed in a nonsterile product, with additional limits on the number of Enterobacteriacae; the preservative efficacy test demands that a specified number of log reductions in viable cells added to a preserved product be achieved within a specified time frame. A linear correlation between plate counts (log CFU/mL) and electrical detection times can usually be readily established for individual bacterial and fungal strains, by using either the direct or indirect electrical detection methods (Firstenberg-Eden and Eden 1984; Connolly et al. 1993, 1994; Deak and Beuchat 1993a). Generally about 100 samples showing a complete range of the inoculum sizes over which measurements are required (ideally 10^1 to 10^8 CFU/mL) should

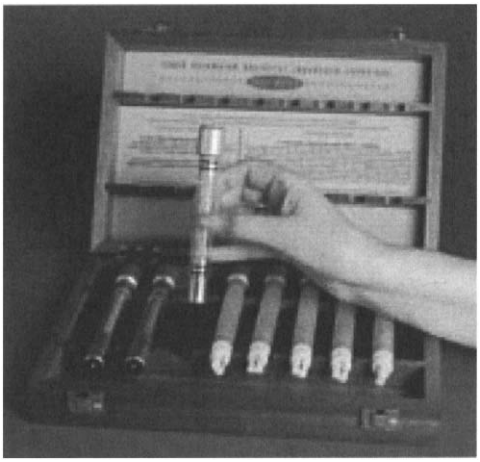
Figure 7.2b An impedance measurement cell being placed into the instrument.



be used in the calibration to ensure its reliability (Firstenberg-Eden and Eden 1984). In some instances, particularly when the expected viable cell number is low, the most probable number (MPN) method is used to estimate viability. Unlike the colony counting and electrical detection methods, the MPN count gives discrete values and thus is a fairly imprecise measure of viability. Even so, a correlation between electrical detection times and MPN has been successfully established (Firstenberg-Eden and Klein 1983; Dupont et al. 1996; Madden and Gilmour 1995). The sensitivity of the electrical detection method is similar to that of MPN, but the former is much faster; for example, one coliform or greater can be detected in a sample within about 16 hours, compared to the traditional 48 hours (Madden and Gilmour 1995). Thus impedance is a quick and easy method for determining the viable count of suspensions containing a single strain of a known organism (or in some cases, a group of similar organisms; see below) for which a calibration curve has been constructed.

The fact that each individual bacterial and fungal strain has its own unique correlation is an obvious drawback when considering impedance as a tool for determining total viable counts of mixed populations, or for counting a particular type of organism within that population. Despite this problem, some progress has been made in establishing reliable correlations for such counts (Firstenberg-Eden and Klein 1983; Firstenberg-Eden and Tricarico 1983; Dupont et al. 1994, 1996). Optimization of the growth media is extremely important. The media should show a rapid change in conductance as a result of cell metabolism by the relevant organisms, while inhibiting the replication of those cells not required for the count. Thus when carrying out a total viable count of fungi, for example, the media should not support the growth of bacterial cells that would

Figure 7.3 Rabit test cells.



interfere with the conductance curves and thereby produce an erroneous detection time. Many media now available have been designed especially for electrical detection and are selective for specific organisms (see below). Another essential parameter is the incubation temperature of the mixed population. Each bacterial and fungal species has its own intrinsic lag time and growth rate (and consequently its own detection time) for the same starting inoculum of viable cells dependent on temperature. This is the major reason for the differing correlations observed for each individual species. In the case of a total viable count, the temperature should be adjusted to encourage a similar growth rate in all the relevant species. Firstenberg-Eden and Eden (1984) demonstrated that reducing the incubation temperature from 37°C, typically used when assessing plate counts of bacteria, to 35°C for electrical detection minimized the differences in generation times among a selection of fecal and nonfecal coliform. As a result, a calibration curve was established to enable impedance to be used in determining the total count of coliforms. In contrast, altering the incubation temperature to promote the rapid growth of a single species while reducing the growth rates of other organisms facilitated the quantification of *Escherichia coli* in a mixed population by conductance (Dupont et al. 1994, 1996). Based purely on the electrical detection times, such calibrations have enabled products to be deemed grossly contaminated, safe for use, or suspect (i.e., at the borderline of safety in terms of the relevant limits). Only the products showing detection times that suggest the intermediate contamination level would require further testing by more conventional methods. The amount of time saved by using this procedure could have a major impact on the manufacture of pharmaceutical products, because both raw ingredients and the final products could be readily assessed for contamination.

If electrical detection is used as a method for determining the total viable count of a pharmaceutical product, it is necessary to ensure that the formulation ingredients do not detrimentally affect the composition of the growth media and/or the conductance measurements. Some formulations may contain highly charged components that could greatly increase the initial baseline conductivity value of the medium, and thus cause problems in detecting significant changes in conductance. The addition of solid material may also affect conductance measurements, because of sedimentation around the electrodes (Dupont et al. 1994). If such problems occur, the indirect electrical detection method could be a useful alternative (Owens et al. 1989).

A further problem, highlighted by Connolly et al. (1993, 1994), is the effect on detection times after the exposure of viable cell suspensions to biocidal agents. Although impedance is able to detect biocidal activity (i.e., samples removed from bacterial suspensions exposed to a biocide show an increase in electrical detection time that coincides with a decline in the numbers of CFU), the correlation of log CFU/mL with detection time in a biocidally treated population is different from that of an untreated suspension. Furthermore, each biocide produces a different correlation, none of them parallel to that of the untreated population, and may not show repeatability between successive experiments (Sheppard 1996). The detection time for a particular inoculum size of viable cells is always increased after exposure to biocidal agents. The principal reason for this is believed to be an extended lag phase in the remaining viable population as a result of sublethal injury (Mackey and Derrick 1984; Sheppard 1996). Clearly, if detection time was used to determine the viability of a stressed population using a correlation established from freshly harvested cells, the viability of the stressed population would be underestimated. This obviously has many serious implications for the usefulness of impedance as a direct alternative to colony counting, especially because in nearly all cases where viable counts of microorganisms are required, the cells may have first been exposed to some form of stress.

Testing for Biocidal Activity

Impedance can be useful for both qualitative and quantitative analysis of antimicrobial activity. A selection of isolates of *Staphylococcus aureus* and gram-negative bacilli was tested for susceptibility to a range of antibiotics by using both impedance and a standard method (Hogg et al. 1987). The impedance method involved incubating inocula of cells with high and low concentrations of the antibiotics (chosen by reference to typical minimum inhibitory concentration [MIC] ranges for the antibiotics) and comparing the growth of the treated cell populations, as reflected in the conductance curves, to that of an untreated suspension. The organisms were categorized as sensitive to the antibiotics if there was no change in the conductance of the media (i.e., no detection times) at both test concentrations, resistant if at both concentrations the conductance curves were similar to that of the control, and of intermediate sensitivity if there was growth at the lower antibiotic concentration but no growth at the higher one. The MIC of the antibiotics for each isolate was obtained by determining the lowest concentration that inhibited growth of the bacteria on agar plates containing serial twofold dilutions of the antibiotic. By the standard method, the isolates were then deemed resistant if the MIC was greater than the high test concentration used with the impedance method, sensitive if the MIC was less than the low test concentration, and of intermediate sensitivity if the MIC was between those of the two test concentrations. Excellent correlation was achieved between the two methods except when *Pseudomonas* isolates were used; Hogg et al. (1987) felt that this could be a result of the poor conductance changes observed with *Pseudomonas,* even with the untreated suspension, a problem that might be rectified by using an alternative medium. Another study has also demonstrated the usefulness of electrical detection in identifying clinical strains of *Staphylococcus aureus* resistant to an antibiotic (Wu et al. 1997). The results obtained showed agreement with those of the disk diffusion method carried out on agar.

Quantitative measurement of antibiotic activity, in terms of determining parameters such as the MIC (Johansen et al. 1995; Zhou and King 1995) and the postantibiotic effect (PAE) (Gould et al. 1989; MacKenzie and Gould 1993), can be successful carried out by electrical detection. Rather than observing a series of tubes for turbidity 24–48 hours after inoculating them with cells and a known concentration of antibiotic, one can establish the MIC by continually monitoring cell replication by using the impedance technology. There is no detection time for the cell populations in which total inhibition of growth, or death of the cells, has occurred. Where no detection time is obtained, the contents of the electrical detection cell can be spread onto agar to establish the minimum biocidal concentration (MBC) (Johansen et al. 1995).

The PAE is the period of time after complete removal of an antibiotic during which there is no growth of the target organism (Zhanel et al. 1991). It is usually quantified by calculating the time difference for an untreated control suspension to produce a one log increase in viable cells compared to the pretreated cell suspension (Craig and Gudmundsson 1991). Rather than frequent plate counts over a period of hours, an electrical detection instrument can be used to compare the growth of the untreated and pretreated bacterial suspensions by means of conductance measurements. An equation has been derived to enable the conductance curves to be used directly in calculating the PAE (Gould et al. 1989). This automated method is highly suitable for the replication of experiments, as it is faster and less labor intensive than viable counting. It is also comparable with alternative techniques (MacKenzie et al. 1993, 1994). In addition, the information gained, compared with the more traditional methods, is better because it indicates which phase of growth (lag phase or log phase) is most affected by prior exposure to antibacterial agents.

Qualitative assessment for antimicrobial activity in which sensitivities are achieved within 4–6 hours (Hogg et al. 1987) could prove useful in the pharmaceutical industry for rapid screening of novel compounds to discover bacteriostatic and fungistatic activity. Because all the electrical detection instruments have a large sample capacity, many different species could be tested simultaneously. Further quantitative tests such as elucidation of MICs, MBCs, and PAEs could then be limited to those compounds of interest, Moreover, analysis of the conductance curves frequently provides more information on the interaction of the antimicrobial agents with the organisms or the effects on cell populations of exposure to biocidal compounds than can be deduced from standard plate counts. A series of increasing concentrations of a biocide up to its MIC, for example, produced capacitance curves showing distinct differences from that of an untreated control (Zhou and King 1995). This suggests that at sub-MIC values, the biocidal agent was having some effect on the growth characteristics of the bacterial population. Similarly, a study of conductance curves of various *Escherichia coli* strains grown in broth containing different antibiotics illustrated the potential problems of isolating the pathogenic strain of *E. coli* (O157). High detection times for some O157 strains indicated that the antibiotics were adversely affecting their growth. The current method for detecting this strain of *E. coli* is to streak it on agar plates containing a combination of antibiotics, a method that this study suggested could result in the reporting of false negatives (MacRae et al. 1997).

The impedance technique has been assessed for determining preservative efficacy in pharmaceutical products (Connolly et al. 1993, 1994). Attempts were made to relate electrical detection with viable counting so that electrical detection could be employed in the Preservative Efficacy Test (approved by the British Pharmacopoeia 1993) as an alternative method of enumeration. Although, as previously mentioned, correlations between detection times and plate counts could be readily obtained for all the untreated bacterial and fungal strains required by the British Pharmacopoeia, the correlations were

significantly altered after the cells were treated with biocides. In addition, it was noted that the recovery of *Pseudomonas aeruginosa* from a cream formulation preserved with methyl parabens produced a correlation different from that of similar cells exposed to methyl parabens while suspended in phosphate-buffered saline. Therefore a quantitative assessment of kill by preservatives which can be directly related to log reductions in viable counts would appear to be impractical. Numerous correlations for the various preservatives, alone and in combination, for a range of formulations would have to be established.

Despite these problems impedance did show a much better dose response to biocidal agents compared to two other rapid methods analyzed, ATP bioluminescence and the direct epifluorescence technique (Connolly et al. 1993). Therefore, impedance could be useful for making a quick decision on which preservative systems may be suitable for newly formulated products. Comparing the electrical detection times of samples taken at selected time intervals from preserved and unpreserved products challenged with relevant organisms would indicate whether the biocide(s) had had any effect on the organisms. Little or no difference in the detection times between the products would suggest poor activity, whereas a large increase in the detection time of the preserved product could be an indicator of preservative action. These successful preservative systems would then be eligible for further tests. A screening program such as this could greatly reduce the amount of work required and the expense involved in choosing suitable preservative systems. It might also enable a greater range of preservative combinations to be evaluated at the preliminary stages, so that the most desirable one could be determined. In addition, the preservatives could be assessed against a wider selection of relevant organisms (e.g., environmental isolates as well as laboratory strains) than is currently possible, given the vast work load inherent in the plate counting method (Connolly et al. 1994).

A further suggestion is to use impedance in stability testing (Connolly et al. 1994). Detailed quantitative data are also unnecessary in these tests, because large numbers of identical samples are stored and analyzed periodically, to ensure that preservative activity has been maintained during storage. Therefore the kill curve in terms of detection times for a particular organism should be no different for a product inoculated immediately after preparation and one that is inoculated with the same number of organisms after storage for 3 months or more.

Testing for biocidal activity is important not only for the development of new antibiotics and in ensuring that multiple dose formulations are adequately preserved; it is also necessary for quality control to monitor the efficacy of the disinfectants used throughout the manufacturing facilities. The formation of biofilms inside manufacturing equipment, particularly in pipes, and on other surfaces where production is carried out are a major hazard for the industry. Biofilms form when microorganisms are able to adhere to and colonize surfaces to form complex structural layers. As a result they can exhibit greater resistance to biocidal agents than suspended cells (Gibson et al. 1995). Currently the assessment of disinfectants against biofilms is carried out as a suspension test, the cells being removed from the surface prior to testing. Such methods cause disruption of the biofilm, and therefore its enhanced resistance to the disinfectant may not be taken into account. The impedance approach allows the disinfection procedure and the detection of surviving cells to be carried out while the biofilm is still surface bound, ensuring that the physiological conditions of the bacteria are maintained (Holah et al. 1990; Dhaliwal et al. 1992; Johnston and Jones 1995). This method involves growing biofilms on disks of relevant material and then placing them into the disinfecting solution for the required time. After the disks are neutralized, each one is added to an electrical detection cell containing a suitable growth medium and monitored for a detection time. This method has been shown to be more reliable than determining viable counts, which involves removing the bacteria from the surface of the disk after treatment and adding them to agar, for observing resistance to disinfectants (Johnston and Jones 1995). Detection times indicating the presence of viable cells were observed, although no recovery was seen on agar, after treatment of equivalent biofilms with disinfectants. Therefore, the electrical method is a more sensitive test than the standard method, because detection is possible down to a single survivor. This is of great importance, because low numbers of surviving cells are capable of re-establishing the biofilm. The electrical method is also faster; results are obtained within 24 hours rather than several days. Thus, this method provides greater confidence that the disinfectant is killing all viable cells, because many replicate analyses can be carried out and the resistance of biofilms attached to different surfaces can be assessed.

Electrical detection has been shown to give a higher estimation of the number of viable cells in a biofilm than alternative methods. Flint et al. (1997) demonstrated that, on the basis of a calibration curve of plate counts against detection times for planktonic cells, the detection times of untreated biofilms suggested viable counts two logs greater than the plate counts achieved after swabbing or epifluorescence microscopy counts. (To show that the calibration produced with planktonic cells was suitable for use with the biofilms, it was demonstrated by means of conductance curves that the growth parameters of biofilms were the same as planktonic cells.)

Instead of assessing disinfectants against biofilms on a purely qualitative basis by determining whether they can produce total kill (i.e., no detection time within 24 hours) or not (Dhaliwal et al. 1992) some workers have attempted to quantify the amount of kill. Calibration curves for detection times with plate counts (Johnston and Jones 1995) or epifluorescence microscopy counts (Holah et al. 1990) for untreated cell populations have been used to estimate the viability of biofilms after treatment with disinfectants. One study used such counts to determine if a selection of commercially available disinfectants could produce a 5 log reduction in viable count within 5 minutes of exposure (Holah et al. 1990). The ability to produce this

amount of kill was shown to be dependent on the mechanism of action of the agents and the concentrations used. However, in view of the fact that detection times for viable cells in suspension after exposure to biocides are different from those of untreated cells (Connolly et al. 1993, 1994), it is probably inappropriate to measure the decline in viability of a biofilm on the basis of detection times converted to viable cell numbers by using a calibration established with untreated cells.

Sterility Testing

As an extension to the methods described for determining the efficacy of biocidal agents, impedance technology could be used as an analytical method for establishing product sterility. It has been evaluated recently as an alternative method for determining the growth of contaminating organisms captured on a membrane, after filtration of the samples, in place of the traditional standard of observing growth media for turbidity after addition of such membranes (Dalmaso and Sofia 1998). Preliminary results suggest that tryptone soya broth incubated at 24°C and 32°C enables the detection of a large range of aerobic organisms, while fluid thioglycollate medium supplemented with the enzymatic system Oxyrase favors the growth of anaerobic species at 32°C. Moreover there was no interference with electrical detection by the membrane or other residues related to the filtration process. Obviously organisms that have managed to survive a sterilization process are likely to be have some damage; although detection times were longer, it was confirmed that the media were still capable of supporting the growth of stressed microorganisms. This study has demonstrated the great potential for impedance technology in routine sterility testing, results being achieved within 4 days rather than the 12 days required for the current method.

Predictive Modeling of Growth

The ability of microorganisms to grow in a product can be greatly influenced by the physicochemical properties of the formulation. Many nonsterile pharmaceutical products—e.g., oral suspensions or creams—contain an abundance of nutrients, and those nutrients encourage the replication of contaminating organisms. Although preservatives will be added to preparations, the manipulation of factors such as the formulation pH, water activity, and storage temperature can also help reduce the risk of product spoilage. Studying the growth rate of an organism at various inoculum sizes in a series of formulations with differing physicochemical properties can enable establishment of a mathematical model to predict its growth in any similar formulation. Because several experimental parameters need to be assessed, the collation of data of adequate quality is a technically demanding task (Deak and Beuchat 1994). Impedance measurement has been reported to be a very efficient method for quantifying specific growth rates and generation times that may be utilized in developing such models (Borch and Wallentin 1993; Deak and Beuchat 1993b, 1994; Dengremont and Membré 1994; Lindberg and Borch 1994). The capacity of the electrical detection instruments means that a large number of factors that potentially affect the growth and activity of microorganisms can be studied simultaneously, enabling polynomial predictive models to be determined. Moreover, a selection of organisms can be evaluated to identify the species showing the greatest tolerance of the stressed environment and therefore being the most suitable for developing a predictive model of spoilage (Deak and Beuchat 1993b). A high correlation has been found between the conductance models that use other predictive models such as absorbance (Lindberg and Borch 1994) or plate counts (Borch and Wallentin 1993; Dengremont and Membré 1994) to determine growth rates. Conductance models could eventually have a major function in predicting the shelf life of products (Deak and Beuchat 1993b).

Detection of Pathogenic Organisms

The European Pharmacopoeia specifies that certain pathogenic organisms must be absent from nonsterile products. The criteria for absence depend on the route of administration of the pharmaceutical formulation and the origins of its raw ingredients. In general the organisms of interest are *E. coli, Pseudomonas aeruginosa, Salmonella* spp. and *Staphylococcus aureus.* Some monographs also require that the product be tested for the absence of Clostridia. As individual strains, all these organisms except Clostridia can be detected by electrical conductance with standard broths such as Iso-Sensitest® broth (*Staphylococcus* and Enterobacteriaceae; Hogg et al. 1987), tryptone soya broth supplemented with sodium pyruvate and sodium chloride (*Salmonella;* Mackey et al. 1984), and modified plate count media or Wilkens and Chalgrens anaerobe broth (*P. aeruginosa* and *S. aureus,* respectively; Connolly et al. 1993, 1994). As contaminants in either raw ingredients or a final product, these organisms are likely to be low in number, other less harmful organisms also being present. Because the standard media can readily sustain the replication of many types of microorganisms, they are of no use when trying to determine the presence or absence of pathogens. Consequently for impedance to play a useful role in detecting pathogens, specialized broths have had to be developed which show both selectivity and a good conductance response when metabolism of the organism of interest occurs.

Salmonella

Because much of the developmental work for impedance has been directed at the food industry, the problem of detecting *Salmonella* spp. has been studied in great detail. The early investigations for suitable media have been outlined in review articles by Blackburn (1993) and Silley and Forsythe (1996). These studies have in general evaluated two basic types of medium. Some were formulated on the premise that *Salmonellae* reduce trimethylamine-N-oxide (TMAO) to trimethylamine (TMA) (Easter and Gibson, 1985; D.Gibson 1987; Pettipher and Watts 1989a); the alternative media were designed to monitor the metabolism of lysine by salmonellas (Ogden 1988; Pettipher and Watts 1989b; Smith et al. 1990).

The first TMAO type media contained selenite-cystine, TMAO, and dulcitol as a carbohydrate source (Easter and Gibson 1985). Although this proved fairly successful, it failed to detect those *Salmonella* strains unable to ferment dulcitol. Mannitol was suggested as a replacement for dulcitol and was shown to enhance the detection of salmonellas (D.Gibson 1987). Pettipher and Watts (1989a) analyzed the suitability of these two media compared to a third one, in which the carbohydrate source was deoxyribose. The number of positive electrical detections for 95 salmonella organisms and forty nonsalmonella organisms, which included *E. coli, Pseudomonas* spp., and *S. aureus,* were compared for each broth. The mannitol broth gave the highest number of positive detections for salmonellas, a rate of 84.2% compared with 77.9% and 74.7% for the deoxyribose and dulcitol broths, respectively. These results concur with those of D.Gibson (1987). However, the mannitol broth also produced the greatest number of false positives with the nonsalmonella organisms (10%) and the deoxyribose broth the least (2.5%). The authors concluded that replacing dulcitol with either of the other carbohydrate sources would improve the sensitivity of the test.

Use of a lysine-based medium also avoids the problems of nondulcitol fermenting salmonella (Smith et al. 1990). Lysine decarboxylase glucose medium (Ogden 1988) was shown to be useful in detecting salmonellas in animal proteins (Smith et al. 1989). This initial formulation was subsequently modified to enhance the conductance response (Smith et al. 1990). Although the new formulation containing lactalbumin hydrolysate, lysine, glucose, sodium chloride, and sodium selenite produced a better conductance curve than the original lysine medium, particularly when adjusted to pH 7, it was not as sensitive in detecting salmonellas as the selenite cystine trimethylamine dulcitol medium of Easter and Gibson (1985). It was therefore suggested that the lysine and dulcitol media should be used in parallel for detecting all *Salmonella* species.

Another lysine-based medium was evaluated for use with impedance by Pettipher et al. (1989b). The electrical detection cells containing lysine-iron-cystine-neutral red media were monitored for conductance changes and also examined after 24 hours for a black discoloration. This color change would result from H_2S production, commonly associated with salmonellas. The production of H_2S was noted for 95% of the 95 strains of salmonella tested, but not found for any of the nonsalmonella organisms (40). Conductance measurements, on the other hand, were able to detect 99% of the salmonella strains, but 22.5% of the nonsalmonella species also gave positive results. Thus, although the conductance method gives better detection of salmonellas than H_2S production, the use of this medium for detecting salmonella in mixed populations by impedance alone would result in a large number of false positive readings.

Examination of the various media indicates that no single medium is able to identify all types of salmonella; thus, as suggested by Smith et al. (1990), lysine and dulcitol media are generally used in parallel for the detection of salmonella. A commercially available impedance method (Malthus), in which a pre-enrichment stage in buffered peptone water containing lysine and glucose is followed by a subculturing step into two selenite-based broths containing either lysine or TMAO and dulcitol which are then monitored for conductance changes, has been accepted by the AOAC International for detecting *Salmonella* species in foods (Blackburn 1993). A collaborative study carried out by several laboratories indicated that this test method was comparable to conventional culture (Gibson et al. 1992). A further study confirmed these findings by comparing the impedance method with two other commercial methods, Gene-Trak® and Salmonella-Tek®, as well as conventional culture (Quinn et al. 1995). Culturally confirmed samples, which included all positives by the conventional technique plus additional positives, noted with the rapid methods, that were subsequently cultured on agar also, indicated that 39.2% of all the samples were contaminated with *Salmonella.* The impedance technique identified 38.4%; Gene-Trak and Salmonella-Tek detected 28.9% and 28.5%, respectively. The conventional method was the least successful; only 25.5% of the samples were found positive. It was suggested that all the rapid methods could be used instead of the conventional technique for routine screening, the impedance method being considered the least labor intensive.

Detecting salmonella by an indirect conductance method is also possible (Bolton 1990). The growth of *Salmonella* spp. in Whitley Impedance Broth was readily detected by monitoring carbon dioxide production, but other organisms also replicated readily in the broth. Bolton (1990) suggested that because the methodology worked, it might be possible to use a conventional selective medium in the system. This was proved correct when it was shown that the indirect impedance method with Rappaport-Vassiliadis broth could distinguish between *Salmonella* species and other closely related organisms (Donaghy and Madden 1993). A further study has indicated that the indirect conductivity method with Rappaport-Vassiliadis broth is a superior method for detecting *Salmonella* spp. than the direct electrical detection method in combination with the two

standard broths (see above). The direct method detected 30% fewer positive samples than the indirect one (Madden et al. 1996).

Escherichia coli

The initial interest for developing an ideal growth medium for *Escherichia coli* was mainly directed toward applying impedance technology to the counting of coliforms (Firstenberg-Eden and Klein 1983). For this reason the different commercially available media formulated to detect coliforms are not selective for *E. coli.* Dupont et al. (1994), however, demonstrated that when 45 different bacterial species were incubated at 44°C in Malthus Coliform Broth, only *E. coli* strains, along with a single strain of *Klebisella pneumoniae,* produced a detection time.

A conductance medium designed specifically for the detection of *E. coli,* containing TMAO and D-glucuronic acid, was first reported by Ogden (1993). This medium was evaluated by screening it against a wide variety of bacterial species (Colquhoun et al. 1995). Good selectivity was demonstrated; only two *Salmonella* species produced an impedance response similar to that of the *E. coli* strains. This methods ability to detect *E. coli* in water samples was compared to the traditional membrane filtration method and Colilert™, a defined substrate medium available commercially. The water samples were filtered through a membrane to concentrate the contaminating organisms and the membrane added to the broth for electrical detection. There was good correlation of the results of all three techniques; the impedance method gave results within 11–14 hours.

An indirect electrical detection method has also shown some promising results for detecting *E. coli* (Timms et al. 1996). When a selection of bacterial species was incubated in membrane lauryl sulfate broth at 44°C, impedance curves were observed only for the *E. coli* isolates and a couple of *Salmonella* species. Furthermore, there were recognizable differences among the impedance curves of these organisms. As with the direct impedance method, indirect electrical detection was much faster than either membrane filtration or Colilert (15 hours compared to 42 hours by membrane filtration or 18–24 hours with the defined substrate medium). The indirect impedance method was, however, slightly less sensitive than Colilert, being unable to detect four or fewer *E. coli* cells in a sample.

Pseudomonas spp.

Some success has been achieved in selectively detecting *Pseudomonas* species with an impedance technique that uses a growth medium supplemented with cephaloridine, fusidin, and cetrimide (Banks et al. 1987). However, this mixture of antibiotics is not always sufficient to inhibit the growth of some Enterobacteriaceae. A study to optimize the medium for detection of *Pseudomonas* spp. using the direct electrical detection method was carried out by Salvat et al. (1997). Four standard media were initially assessed to establish which one produced the best conductance curves. Previous studies had indicated that there can be great variation in the appearance of the conductance curves for *Pseudomonas* species in different broths (Firstenberg-Eden and Tricarico 1983). An assortment of agents reported to enhance the growth kinetic of *Pseudomonas* species and a series of antibiotics believed to be capable of inhibiting potential competitive organisms were added systematically to the chosen broth. A good compromise between growth of *Pseudomonas* and inhibition of other organisms was achieved with Whitley Impedance Broth supplemented with carbenicillin, cetrimide, cycloheximide, diamide, and metronidazole.

None of the *Pseudomonas* species tested with this broth is the pathogen *Pseudomonas aeruginosa,* considered the one of greatest interest in the field of pharmaceutical products. This work does, however, illustrate that it is possible to develop media suitable for use with electrical detection systems which can in general inhibit competing organisms while encouraging the replication of *Pseudomonas.* Whether media can be developed to select among the different species remains uncertain.

Staphylococcus aureus

There is no published literature related to the specific detection of *Staphylococcus aureus* in mixed populations; this organism is generally isolated by using high salt concentrations, which are inhibitory to the majority of other microorganisms. Such a medium is unsuitable for direct electrical detection, but it could be used in the indirect method for selectively monitoring the growth of *S. aureus.* Using the indirect method, Bolton (1990) observed that the growth of two strains of *S. aureus* in Whitley Impedance Broth supplemented with lithium chloride was similar to that seen in unsupplemented media. Addition of sodium chloride to the impedance broth, however, caused a delayed detection of the organisms.

Clostridia

Organism-detecting methods that require anaerobic growth have been reported (Zhou and King 1995; Dalmaso and Sofia 1998), and there are some commercially available products. The growth of *Clostridium perfringens* and *C. sporogenes* in five different media has been assessed by using conductance (Dromigny et al. 1997). Only two broths, fluid thioglycollate medium and Columbia Broth Malthus, produced reasonable conductance curves, although the latter was less effective for detecting the growth of the two *Clostridium* species. However, these broths are nonselective. Detection of growth in a selective broth by using impedance measurements of *C. botulinum* has been reported (A. Gibson 1987). The indirect electrical detection method could prove useful in these circumstances also.

MISCELLANEOUS APPLICATIONS IN DRUG DEVELOPMENT

There have been limited reports of using an electrical detection method during the development of drugs other than those selected or designed specifically for antimicrobial activity. Dale and Edwards (1989a) used the technology to determine the relative toxicities of compounds believed to be useful bioreductive cytotoxic and radiosensitizing agents. Such agents are designed to have selective toxicicity under anaerobic conditions, to act against cells in mammalian tumors that are resistant to radiation treatment, surviving in areas of low oxygen tension. *E. coli* was selected as a microbial model and was grown under aerobic and anaerobic conditions in broths containing increasing concentrations of the drugs. Growth of the bacteria was monitored via conductance measurements, enabling a D_{50} value (the drug concentration that had a detection time twice that of an untreated suspension) for each drug under aerobic and anaerobic conditions to be calculated. A comparison of the relative toxicity under the two growing conditions could then be made. Because this *in vitro* study showed results similar to those of previous *in vivo* studies, Dale and Edwards (1989a) suggested that electrical detection was useful for rapid screening of these cytotoxic agents.

Examining the conductance curves of wild type and mutant strains of *E. coli* in the presence and absence of drugs has led to the elucidation of the mode of action of a cytotoxic drug (Dale and Edwards 1989b) and the development of a screen for mutagenic agents (Forsythe 1990). Alteration in the growth parameters of three mutants with known defects in specific DNA repair in the presence of a drug, compared to a wild type strain, gave an indication of how the function of DNA was impaired by the drug (Dale and Edwards 1989b). Using a similar procedure with a wild type *E. coli* strain and a single DNA-repair mutant, Forsythe (1990) was able to classify a range of drugs as either direct-acting mutagens or bactericidal agents. Changes in the viability of the bacterial suspensions in the presence of the drug were determined via impedance. Direct-acting mutagens were indicated when there was a loss of viability in the mutant population but no change in the viability of the wild type suspension, whereas an equal loss of viability in both cell populations indicated bactericidal activity only.

A further application of impedance was reported by Frenoy et al. (1994). They studied the *in vivo* phagocytosis of a bacterial population infused into an animal model by determining the electrical detection times of blood samples. Using a correlation established between detection times and cell numbers, they calculated the rate of clearance of bacteria from the bloodstream. The technique was used in evaluating the reticuloendothelial system-stimulating properties of a drug administered to the test animals, and it was shown to give results similar to those of the standard in vivo method of carbon clearance.

CONCLUSION

This review has illustrated the large number of potential applications that impedance microbiology has in the pharmaceutical industry. This discipline may be particularly useful in the detection of specific microorganisms in raw ingredients or final products, and it could also be applied to the qualitative analysis of biocidal agents. We emphasize that the technique, whether used in a qualitative or quantitative role, must be thoroughly validated. The literature has, however, provided reasonable detail, so that such validation can be successfully carried out, enabling realization of the full benefits of this highly automated, rapid technology.

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8
Traditional and Automated Rapid Methods for Species Identification and Typing

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Until recently, the major emphasis in determining bacterial relatedness was placed on techniques that assessed one or more phenotypic markers. These included methods such as serotyping, phage typing, biotyping, antibiotic susceptibility testing, and bacteriocin typing. It is now well known that at the bacterial species level, there is sufficient genetic diversity to allow identification of different clones (genetically identical organisms descended from a single common ancestor) by means of molecular typing methods that incorporate genotypic and chemotypic techniques. These can be broadly defined as methods used to physically characterize bacteria on the basis of their DNA composition (genotyping) or production of proteins, fatty acids, carbohydrates, or other biochemical content (phenotyping or chemotyping). The most common genotypic typing methods currently used include chromosomal DNA restriction analysis, plasmid typing, DNA probe—based hybridizations such as ribotyping, pulsed-field gel electrophoresis, and PCR-based methods such as randomly amplified polymorphic DNA (RAPD), rep-PCR, PCR-ribotyping and PCR-restriction fragment length polymorphism (PCR-RFLP). There are also commercially available kits for DNA probing and PCR analysis. Those methods involving PCR primer technology and hybridizations with chemiluminescent DNA probes have been referred to as DNA fingerprinting techniques because the patterns generated are a result of interaction with the genome of the microorganism. The most common chemical typing methods include pyrolysis mass spectrometry (Py-MS), Fourier transform infrared spectroscopy (FTIR), gas-liquid chromatography (GLC), and electrophoretic protein typing. These methods for the most part have been referred to as chemical imaging techniques, because the operational fingerprint or pattern that is generated is based on the interaction with biochemical structures. The exception is electrophoretic protein analysis, where the patterns generated are based on some larger molecular content and are thus generally referred to as molecular fingerprints. Listings of some of the characteristics of bacterial typing methods are in Table 8.1 and Table 8.2.

As technology has evolved, many previously complex processes have been automated, miniaturized, and linked to computer control centers that guide all aspects of the operations, including data analysis. Researchers can now take advantage of technology that was previously available only to well-financed, technically advanced facilities and are now able to generate more timely data at a lower unit cost. Because computer technology now handles the bulk of data storage, processing, and analysis, there is no longer a requirement for in-depth knowledge of statistical methods. Of particular interest to those involved in the biochemical analysis of microorganisms are procedures that have been adapted or are amenable to whole-cell techniques, because they offer all the conveniences of rapid and economical analysis. Huge libraries of customizable computer databases are now available to assist in pattern recognition for detection and identification of microorganisms based on analysis of whole cells, as well as individual genetic elements or chemical derivatives. Industries' need to produce results quickly and reduce turnover times have been a stimulus for advancements. Automated methods are now available for detection, identification, typing, and analysis of biological components or structural changes that result from environmental pressures or extraneous influences. Benchtop versions of sophisticated devices allow for more portability and efficient use of space, which may be precious to some labs. From the standpoint of the pharmaceutical industry, the microorganism is of special interest in the development of biologically active metabolites into new pharmaceuticals, and thus there is a need for rapid and efficient screening of large numbers of microbial cultures (Bevan et al. 1995). In addition, low levels of microbial contaminants in the production process must be detected at the earliest stage possible.

GENOTYPING

Genotyping, which involves the direct DNA-based analysis of chromosomal or extrachromosomal (e.g., plasmids) genetic material, has many advantages over traditional typing procedures (Mazurek 1993; Swaminathan and Matar 1993; Versalovic et al. 1993). The major advantage lies in its increased discriminatory power, i.e., in its ability to distinguish between two closely related strains. Other advantages of genotyping include: (1) DNA can always be extracted from bacteria so that all strains are theoretically typeable; (2) analytical strategies for the genotypic methods are similar and can be applied to DNA from any source; (3) procedures do not generally require species-specific reagents; and (4) it is amenable to automation and statistical data analysis (Arbeit 1995; Bingen et al. 1994).

Restriction Endonuclease Analysis

Restriction endonuclease analysis (REA) was one of the first of the chromosomal DNA-based typing schemes. This technique involves the isolation and restriction digest of chromosomal DNA followed by electrophoresis, through an agarose gel, which effectively separates the DNA fragments on the basis of size. Hundreds of different restriction enzymes exist, each one recognizing

Table 8.1 Characteristics of Genotypic Bacterial Typing Systems

Typing System	Proportion of strains typeable	Reproducibility	Discriminatory power	Ease of interpretation	Ease of performance
Plasmid profile analysis	Variable	Fair to good	Good	Good	Excellent
Restriction endonuclease analysis	All	Very good	Good	Poor	Excellent
Ribotyping	All	Excellent	Fair to good	Very good to excellent	Fair to good
Pulsed-field gel electrophoresis	All	Excellent	Excellent	Excellent	Fair to good
PCR ribotyping	All	Very good to excellent	Good	Excellent	Very good to excellent
PCR restriction digest	All	Excellent	Good	Excellent	Very good to excellent
RAPD	All	Fair to Good	Very good to excellent	Very good	Very good to excellent

Adapted from Arbeit (1995) and Maslow et al. (1993).
Some of the rankings listed above are only from the author's experience and discussions.

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Table 8.2 Major Characteristics of DNA and Chemical-based Methods for Typing or Identification of Microorganisms

Method	Principal application	Sensitivity	Time to completion	Major advantage	Major disadvantage	Cost/sample (US $)	References
Polymerase chain reaction (PCR) and deoxy-ribonucleic acid (DNA) probe kits	Detection and identification from primary cultures	10^4–10^6 CFU/mL, a single colony (1 mm in diameter), or one copy of DNA	16–26 hours, depending on kit	Can conveniently screen out large numbers of negative samples	No viable bacterial cells to go back to	$1–8 depending on kit (most try to stay around the $ 1 mark)	Bailey (1998), Freney et al. (1993), Hill (1996), Stewart and Gendel (1998)
RiboPrinter™	Subtyping of previously identified micoorganisms	1 µL loopful	8 hours	Fully automated	Expense	$45	Product/ Technical literature/ support (1999g)
Whole-cell protein profile (WCPP), lipo-	Microbial typing below species level	Approx. 20–40 mg of cells (wet weight)	2 days	Relatively inexpensive	Labor intensive	?	Costas (1990)

Method	Principal application	Sensitivity	Time to completion	Major advantage	Major disadvantage	Cost/sample (US $)	References
polysacharide profile (LPS), and multi-locus enzyme electro-phoresis (MLEE)							
Pyrolysis mass spectrometry (Py-MS)	Analysis of whole cells to determine the epidemiology of infections due to outbreaks	Single colony (1–10 µg dry weight)	<2 minutes from pure culture	Speed and automation	Expense of equipment	$2.40	Goodfellow et al. (1997)
Fourier transform infrared spectroscopy (FTIR)	Analysis of whole-cell components for microbial identification and typing	Microcolonies 40 µm in diameter (approx. 104 cells)	< 1 hour from pure culture	Speed and automation	Expense of equipment	$2	Helm et al. (1991 b), Naumann et al. (1991)
MIDI microbial identification system (MIS)	Analysis of whole-cell fatty acid methyl esters for microbial identification and typing	2.4×10^{10} cells (approx. 4 mg dry weight)	<2 hours from pure culture	Fully automated	Not as discriminatory for typing; must adhere strictly to protocol for preparation	$1.30	Basile et al. (1998a), Product/ Technical literature/ support (1999c)

a particular and different nucleotide sequence (Lewin 1991). The enzymes most commonly employed in REA recognize nucleotide sequences between 4 and 6 bases and are referred to as frequent-cutting restriction enzymes, as opposed to low-frequency cutters, which recognize a set of 6 or 8 bp (base pairs). The restriction fragments generated usually range in size from 1,000 to 20,000 bp and are visualized under UV light when stained with ethidium bromide. The resulting DNA fingerprints or profiles can be compared between and among strains.

Because of the high specificity of restriction enzymes, a reproducible pattern of fragments is obtained after complete digestion of the chromosomal DNA with a particular enzyme. The observed differences in the banding profiles of two particular isolates is referred to as a restriction fragment length polymorphism (RFLP) and may result from subtle changes in the chromosome from minor insertions, deletions, or rearrangement of the DNA. The major disadvantage of this technique is the complexity of the banding patterns as a result of the numerous bands generated from frequent cutting of whole chromosomal DNA (Maslow et al. 1993) and thus the subjectivity of unaided, manual analysis.

Plasmid Typing

Plasmid typing is one of the oldest and simplest of the genotypic typing methods. Plasmids are generally found as small, circular pieces of double-stranded DNA that exist as separate entities, independent of the bacterial chromosome. They are self-replicating bodies that usually encode product(s) and/or function(s) that modify the phenotype of the cell. They generally carry antibiotic resistance factors and may range in size from 1.5 kilo bases (kb) to about 300 kb. These extrachromosomal elements are an integral part of the genetic makeup of most bacteria; however, some strains may not carry plasmids, or their plasmids may be lost in bacterial replication. Thus, plasmid typing, although simple and convenient, may not be a reliable long-term typing method.

In general, plasmid typing involves a procedure that is expected to isolate, if present, only the plasmid content of the cell (Threlfall and Woodford 1995). The extrachromosomal elements are separated by standard gel electrophoresis and stained with ethidium bromide to determine their presence, number, and molecular size. This procedure is generally supplemented with a restriction digest to linearize the plasmids and differentiate same-size elements. The latter is necessary because plasmids of varying sequences may coincidentally have a similar size and therefore not be differentiated by standard molecular sizing on the agarose gel. Thus, by using a particular restriction enzyme (or set of enzymes) on all sample preparations prior to gel electrophoresis, one would expect to cut similar-sized plasmids of varied sequences into distinct fragments that would migrate differently on the gel. The resulting banding patterns could then be accurately evaluated.

Plasmid profiling has been used both in the investigation of foodborne or diarrheal diseases (Wachsmuth et al. 1991) and an epidemiologic tool in clinical microbiology labs (Pfaller and Hollis 1989).

Ribotyping

Ribotyping is one variation of several methods that may be grouped under the general heading of southern hybridization fingerprinting (Schmidt 1994). In southern hybridization methods, a labeled probe is used to identify a complimentary DNA molecule among other DNA fragments in a gel. This can involve, among other things, using probes containing toxin genes (Swaminathan and Matar 1993) or insertion sequences, also referred to as IS typing (Mazurek 1993; Soldati and Piffaretti 1991) or bacteriophage DNA (Samadpour et al. 1993). Ribotyping is simply the use of DNA probes to recognize ribosomal genes. Ribosomal RNA (rRNA) is present in all bacterial cells and is composed of three highly conserved subunits, 23S, 16S, and 5S rRNA, that have only slight variations among and between species. In a typical bacterial cell such as *E. coli,* rRNA makes up approximately 82% of the total RNA content. While most bacterial genes are present in only one copy, rRNA (or rrn) operons can be present in anywhere from 2 to 11 copies per bacterial cell. An operon is a group of contiguous genes that are made by a common promoter and which can all be subject to control by one gene product (e.g., a protein). Because the rRNA genes are very similar in many different bacteria, differing only in their copy number and location within the chromosome, they make an ideal target for species differentiation, and the more copies of an rrn operon present in a particular bacteria, the more discriminatory ribotyping will be.

Initially ribotyping is similar to REA analysis in that bacterial chromosomal DNA is isolated, cut up into many small fragments by using restriction enzyme digestions, and separated by electrophoresis in an agarose gel. At this point however, the separated DNA fragments are transferred either electrophoretically or by capillary action from the gel onto a piece of nylon or nitrocellulose membrane (Bingen et al. 1994; Lipuma and Stull 1991). The membrane provides a solid support for DNA probing and hybridization, nylon being preferred over nitrocellulose for ease of handling. The DNA on the membrane is then covalently bound through heating or UV cross-linking. For the probe itself, commercially available *E. coli* 16S+23S rRNA was most often used historically. It is now more common, however, for researchers to use either a synthetic oligonucleotide made from the 16S or 16S+23S gene sequences or PCR-amplified cDNA (cloned) made directly by reverse transcription from a recombinant plasmid in which the rrn DNA has been inserted (Bingen et al. 1994). Radioactive probe labeling used to be the most common, but it has now been replaced by nonisotopic cold-labeling systems, some of which are commercially available (Bingen et al. 1994) (i.e., a chemiluminescent ribotyping scheme using digoxigenin-labeled rDNA [DNA that has been made by reverse transcription of rRNA]). The reagents used in hybridization protocols tend to be specific for the labeling system used, but they are all based on common principles of blocking nonspecific sites and washing away nonhybridized components. After hybridization with the labeled probe, a development stage follows in which the membrane is exposed to autoradiography film. Each fragment of bacterial DNA corresponding to a part of a ribosomal gene will have a labeled probe attached and thus will leave an exposed band on the film. This effectively creates a fingerprint pattern containing approximately 1 to 15 bands that can be easily compared among isolates. Among the major advantages of ribotyping is its consistent reproducibility and the conservation of ribosomal genes among bacteria that allows for the use of a universal probe. However, this process generally takes 2–3 days to get a final banding pattern or genetic fingerprint that can be compared with other samples to differentiate or group strains.

Initially regarded strictly as a typing method, ribotyping has been developed into an efficient identification tool with the advent of the DuPont RiboPrinter™ and computer database of ribopatterns. The RiboPrinter™ Microbial Characterization System combines all aspects of traditional ribotyping in a hands-off procedure. The only requirement is to grow the culture to be analyzed. The whole process can be divided into 7 stages, including sample preparation of bacterial cultures, DNA preparation, DNA separation and transfer, membrane processing using a sulfonated 16s rRNA probe from *E. coli,* detection of the chemiluminescent fragments by means of a CCD (charge-coupled device) camera, analysis by comparison to previous ribotype patterns existing in the computer database, and optional data manipulation and sorting. Only the first and last stages require manual operations. By using this RiboPrinter Microbial Characterization System, 32 strains in four batches can be started within an 8-hour working day, all results being obtained within 16 hours (Product/Technical literature/support 1999f). Relatively new to the field of molecular typing, the RiboPrinter™ has so far been applied to the identification and characterization of food-borne pathogens (e.g., *Salmonella* [Oscar 1998] and *Listeria* [Ryser et al. 1996]), medically important nosocomial bloodstream pathogens such as *Staphylococcus aureus, Enterobacter cloacae* (Pfaller et al. 1997), *E. coli,* and *Pseudomonas* (Pfaller et al. 1996), investigation of reservoirs harboring bacterial pathogens (Batt 1997) and the diversity or distribution of *S. aureus* and *Streptococcus* among dairy herds (Rivas et al. 1997). The complete system sells for $175,000 US and results are obtained within 8 hours at a cost of $45 US per bacterial isolate from as little as a 1 µL loopful of culture (Product/Technical literature/support 1999f). The cost may appear high compared to other typing schemes, but one must consider the type of information being obtained and the relative labor savings gained by completely automating all aspects of protocol and analysis.

PFGE Typing

Pulsed-field gel electrophoresis (PFGE) typing has proven to be a very discriminating and reproducible typing method. PFGE tries to eliminate some of the problems that occur with restriction enzyme analysis on a micro level (microrestriction analysis), whereby, as stated previously, hundreds of different bands can appear. PFGE is a form of macrorestriction analysis in which infrequently cutting restriction enzymes are employed (ones that will recognize specific 8-base or 6-base sequences rather than 4 bases). Therefore, only a small number of very large fragments are generated (5 to 20 fragments ranging in size from 10 to 800 kb) which can be easily resolved on agarose gels.

The migration of macrorestricted fragments in the pulsed-field gel may be affected by many different factors, including electric field strength, field angle and shape, pulse time, agarose type and concentration, and ionic strength and temperature of buffer system. For a detailed description of these factors, or PFGE in general, see the review by Gemmil (1991) and the book by Burmeister and Ulanovsky (1992). A recent protocol has also been published (Anonymous 1998a).

Two noteworthy elements of PFGE make it unique. The first stems from the fact that DNA fragments larger than 25 kb are not resolved efficiently by conventional electrophoretic means. Thus, a modification involving periodically changing the orientation of the electric field across the gel is required. This has been accomplished by different techniques, but the two most common are field-inversion gel electrophoresis (FIGE) and fleld-angle alternation-based methods such as contour-clamped homogenous electric field (CHEF) gel electrophoresis. FIGE is the least complicated and least expensive and appears to be the best method for resolving DNA molecules ranging from 100 to 200,000 bp (Burmeister and Ulanovsky 1992; Gemmil 1991). In this system, the electric field is simply alternated between the forward and reverse direction, the forward pulse being set up to three times longer than the reverse one. The CHEF system appears optimal for the separation of DNA molecules as large as 3×10^6 bases (Product/Technical literature/support 1999b) and employs an arrangement of hexagonal electrodes that typically generate uniform electric fields at 120° to each other; however, this angle can be varied in newer systems. This causes the DNA fragments to move along a straight line with little or no distortion. Built-in features typically allow the operator to select the correct running conditions for a particular application.

The second unique feature of PFGE is in the preparation of the whole chromosomal DNA extracts prior to digestion with the appropriate restriction enzyme(s). Because whole chromosomal DNA prepared in solution is subject to spontaneous shearing into random fragments, intact microorganisms are embedded in agarose plugs to aid in the preparation of intact high-molecular-weight chromosomal DNA. The cells in the plug are lysed by a suitable lytic agent such as lysozyme or sodium dodecyl sulfate (SDS), and then the contaminating cellular proteins are digested with proteinase K. Several washes prior to using restriction endonucleases are necessary to remove the former two agents, both of which can inactivate the restriction enzymes. The isolated DNA is then digested in situ with a low-frequency cutting enzyme.

Some of the more common problems encountered with PFGE include: (1) inadequate cell lysis or incomplete protease digestion, causing incomplete restriction digestion of the DNA, leading to smearing of the gel at the highest DNA bands; (2) the production of DNAse by organisms such as *Campylobacter jejuni,* leading to degradation of DNA before PFGE (Gibson et al. 1994); and (3) ineffective washing of plugs prior to the restriction digest. Another potential problem results from too little or too much DNA being applied to the gel, leading to lanes of varying intensities and potential interpretation problems.

PFGE has now been applied to a wide range of organisms and has become the genotypic method of choice for many because it is very discriminatory and reproducible, and theoretically all microorganisms can be typed. PFGE has recently been used to help in the investigations of widespread food-borne outbreaks involving *Salmonella* (Van Beneden et al. 1999), *Listeria monocytogenes* (Anonymous 1998b; Proctor et al. 1995), and *E. coli* 0157:H7 (Barrett et al. 1994). It also has recent application in the typing of mycobacteria (Philipp et al. 1998) and medically important nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Mueller-Premru and Muzlovic 1998) and vancomycin-resistant enterococci (VRE) (Singh-Naz et al. 1999).

PCR-Based Typing Systems

One can think of the polymerase chain reaction (PCR) simply as an in vitro method for the enzymatic synthesis of specific DNA sequences. PCR consists of two synthetic oligonucleotide sequences, about 20 nucleotides in length, known as primers. These short pieces of single-stranded DNA are designed to be homologous to a complementary region on each of the two strands of the chromosomal DNA double helix that flank the region of interest and are necessary to initiate synthesis of a new DNA strand (Lewin 1991). A forward and reverse primer must be designed to amplify each strand of the double helix, moving from 5 prime (5') end of strand to 3 prime (3') end, and obtain the logarithmic amplification that marks PCR. The ability to design adequate primers thus presupposes some knowledge of the DNA sequence being amplified. Virulence genes are often the target of PCR amplification because of their uniqueness and importance in pathogenesis (Swaminathan and Matar 1993).

There are three major steps to the PCR once all reaction components have been added to the mixture (template DNA, primers, dNTPs [dioxyribonucleotide tri-phosphate], Mg_2Cl, *Taq* DNA polymerase). First, the double-stranded chromosomal DNA must be denatured into two single strands to expose the homologous primer binding site. This is usually accomplished by heating at 94°C. Second, the added primers must be allowed to attach or anneal to the complementary region on the target DNA. Annealing temperatures vary depending on the specificity desired (i.e., the stringency of the reaction) and the melting temperature of the primer (the temperature at which the primer will no longer bind efficiently to the template DNA). The third step involves the actual extension and duplication of the region of interest with the help of a heat-stable DNA polymerase and the four deoxyribonucleoside triphosphates (Lewin 1991). Each complete cycle of denaturing, annealing, and extension, lasting about 5 minutes, doubles the amount of DNA made in the previous cycle. Typically 20 to 30 cycles are required for effective DNA amplification, approximately a millionfold amplification of target DNA.

Randomly amplified polymorphic DNA (RAPD) is one of the newer genotypic methods based on PCR (Williams et al. 1990). RAPD is probably the simplest and quickest of the genotypic methods and has several advantages. The most significant is that no prior knowledge of the target DNA sequence is required. RAPD differs slightly from the "normal" PCR reaction in that the primers used are generally short (only 9 or 10 bp or nucleotides) and the sequences are chosen at random. Because the primer is a short oligonucleotide, there will theoretically be many sites complementary to it scattered throughout a genome. In addition, the annealing temperature for RAPD is typically much lower than for a normal PCR, allowing a single primer with no known homology to the genome to anneal at sites for which the match is imperfect. When two such adequate but imperfect annealing sites occur close enough (within about 200 to 2,000 bp of each other) and in the correct orientation (5' to 3') on opposite DNA strands, the sequence between the sites can be amplified. Every site that is amplified will eventually lead to the appearance of a band on the stained agarose gel. The actual source of the band differences or polymorphisms between different strains can occur as a result of the deletion of a priming site, the insertion of DNA that moves priming sites too far apart to support amplification, or DNA insertions or deletions that change the size of a DNA segment without preventing its amplification. Although one can use purified DNA for RAPDs, the simplicity of this technique becomes evident when using whole bacterial cells (Mazurier et al. 1992), which are usually lysed by using a hot-start technique whereby the sample is preheated in the PCR machine before the DNA *Taq* polymerase is added.

Because RAPD amplification is a mixture of artifactual bands combined with true polymorphisms, the major concern has been with its reproducibility. Recent work, however, with specific bacteria has shown that the method can be reproducible under carefully controlled conditions (Farber and Addison 1994; Neiderhauser et al. 1994). As a general rule of thumb, polymorphic DNA bands should be scored as such only if they are observed in repeat amplifications involving different DNA preparations and if their presence or absence is not affected when the amount of target DNA is doubled. The numerous factors cited for causing variations in the fingerprint patterns obtained upon repeated trials include: the purity and composition of template DNA and primers used; the concentration of the individual components; most notably the magnesium content; the quality and source of DNA *Taq* polymerase; and the model of PCR machine employed. See Table 8.3 for a list of references about these issues.

Another PCR-based method, referred to as *repetitive element (rep) PCR,* targets repetitive elements in the bacterial genome. It takes advantage of the fact that bacteria are known to contain exact copies of DNA sequences scattered throughout the genome (Versalovic et al. 1991). These interspersed repetitive DNA elements (rep elements) are separated by various distances within the chromosome, depending on the individual bacteria. Because of the variable nature of these elements, any PCR amplification of the genomic region between these repeats will generate DNA fragments of various sizes. The PCR products can then be separated with traditional agarose gel electrophoresis to produce species- or strain-specific DNA fingerprint patterns. Common repetitive chromosomal targets that have been demonstrated in gram-negative bacteria are the enterobacterial repetitive intergenic consensus (ERIC) and the repetitive extragenic palindromic (REP) sequences; however, these targets have been found useful in fingerprinting all eubacterial genomes (Versalovic et al. 1991). Recently, ERIC and REP primers were found to have a high index of discrimination with the gram-positive bacterium *L. monocytogenes* and were able to cluster strains on the basis of origin of isolation. Further discrimination was possible within each cluster based on

Table 8.3 Experimental Parameters Found to Influence RAPD analysis

Parameters observed to influence RAPD profiles and reproducibility	References citing confirmatory observations
[Mg]	Ellsworth et al. (1993), Gao et al. (1996), Hilton et al. (1997), Khandka et al. (1997), Meunier and Grimont (1993)
[Taq]	Meunier and Grimont (1993), Penner et al. (1993)
Natural contamination	Bottger (l990)
Source	Meunier and Grimont (1993), Schierwater and Ender (1993)

Parameters observed to influence RAPD profiles and reproducibility		References citing confirmatory observations
[DNA]		Gao et al. (1996), MacPherson et al. (1993), Meunier and Grimont (l993)
Preparation		Khandka et al. (1997), Micheli et al. (1994), Hilton et al. (1997)
Secondary structure		Caetano-Anolles (1993)
[Primer]		Khandka et al. (1997), MacPherson et al. (1993), Meunier and Grimont (1993)
Length		MacPherson et al. (1993), Williams et al. (1990)
G+C content		MacPherson et al. (1993), Williams et al. (1990)
Importance of primer selection		Berg et al. (1994), He et al. (1994), Welsh and McClelland (1990)
Ratio **Primer/Template**		Caetano-Anolles (1993), del Tufo and Tingey (1994), Ellsworth et al. (1993), MacPherson et al. (1993),
Template/DNA polymerase		Khandka et al. (1997)
Reaction buffer and pH		Hilton et al. (1997)
Thermocycler	-Annealing temperature	Ellsworth et al. (1993), Meunier and Grimont (1993), Welsh and McClelland (l990),
	-Cycle number and time	del Tufo and Tingey (1994), MacPherson et al. (1993), Meunier and Grimont (1993)
	-Extension time	Van Leuven (199l), Wilson (1994)
	-Ramping	Cocconcelli et al. (1995)
	-Model	del Tufo and Tingey (1994), MacPherson et al. (1993), Meunier and Grimont (1993), Penner et al. (1993)
	-Temperature across block	Van Leuven (1991)
RNA contamination		Ellsworth et al. (1993), Micheli et al. (1994),
Type of gel		Berg et al. (1994)
Interlaboratory	-due to *Taq*	Meunier and Grimont (1993) Schierwater and Ender (1993)
	-due to thermocycler	MacPherson et al. (1993), Meunier and Grimont (1993), Penner et al. (1993)
Shown not to affect profiles		
Reaction buffer components and pH (including additives—gelatin, DMSO)		Gao et al. (1996), Meunier and Grimont (1993)
Subculturing		Gao et al. (1996)
Plasmid content		Elaichouni et al. (1994)

Note: Items in **bold** represent those factors cited most often as contributing to variability.

Adapted from Tyler et al. 1997. *Journal of Clinical Microbiology,* 35. (Used with permission of American Society of Microbiobgy, Washington, D.C.)

serotype (Jersek et al. 1999). In addition, the method has been extended as a means of identifying particular species of eucaryotic microorganisms (Judd et al. 1993; van Belkum et al. 1992). The rep-PCR technique is quite reproducible and has moderate discriminatory power.

PCR—restriction fragment length polymorphism (RFLP) is a genotypic method that, in contrast to standard southern hybridizations, is rapid and only involves individual genes or gene clus ters (Arbeit 1995; Swaminathan and Matar 1993). Popular targets of PCR-RFLP are virulence genes (Samadpour 1995), because they are commonly associated with only pathogenic strains of a particular species. This technique incorporates the principles of PCR and REA in one rapid method. Initially, a target gene is amplified using known primers to a particular virulence region. The resulting amplicon (usually 1 to 2 kb) is then digested with a suitable restriction endonuclease, and the fragments are run out onto an agarose gel and stained with ethidium bromide to detect polymorphisms in the gene. Theoretically, any known region of interest can be amplified by PCR and then examined by means of enzyme restriction (e.g., genes coding for flagella [Madden et al. 1998]). Even though prior knowledge of the DNA region of interest is needed and the discriminatory power of the method tends to vary substantially depending on the different species, loci, and restriction enzyme, this technique has the advantage of speed, simplicity, and reproducibility. Investigators have shown the utility of this method by using it not only for typing, but also for strain identification when applied to the ribosomal RNA region or some species-specific gene sequence. Organisms such as *Mycobacterium* spp. (Taylor et al. 1997), *S. aureus* (Schmitz et al. 1998), *Helicobacter pylori* (Dzierzanowska et al. 1996),

Campylobacter (Madden et al. 1998), and *L. monocytogenes* (Vaneechoutte et al. 1998), have all been typed with this method.

PCR-ribotyping, a relatively new genotypic typing method, is also based on the PCR reaction (Jensen et al. 1993; Kostman et al. 1992). The method takes advantage of the heterogeneity found within the spacer regions that exist between the rRNA subunits (16S, 23S, and 5S rRNA) of prokaryotic microorganisms (Campbell et al. 1993; Loughney et al. 1982), most notably the 16S-23S intergenic spacer region. The spacer regions separate the rRNA subunits and may show a large degree of sequence and length variation at both the genus and species level (Campbell et al. 1993; Loughney et al. 1982). Because most bacterial genera contain multiple copies of the rRNA operon, multiple bands of varying lengths may be obtained from a particular strain after amplification with primers designed to target the conserved regions flanking the spacer sequences in different ribosomal operons. As discussed under ribotyping, a distinct advantage of involving the highly conserved rRNA gene as a target in amplification is the fact that universal primers may be designed that will work across several genera of bacteria (Jensen et al. 1993). However, as with traditional PCR, one must have some knowledge of the conserved nature of the rRNA sequence of the particular organism one is trying to type in order to know if primers are adequate. This technique has been used to type organisms such as *S. aureus, Enterococcus faecium, E. coli, Enterobacter* spp., and *L. monocytogenes* (Kostman et al. 1995; Sontakke and Farber 1995).

Although it has been shown that the greatest variation lies within the 16S-23S spacer region and considerably less in the 23S–5S region, researchers have found that increased discrimination can be obtained in some organisms by employing primers that span the entire 16S—5S region, so that the spacer region between the 23S and 5S rRNA is also amplified (Sontakke and Farber 1995). Another approach has been to use restriction endonucleases to cut the amplicons arising from the initial amplification (Kostman et al. 1992) in order to reveal hidden polymorphisms and thereby increase the discriminating ability of PCR-ribotyping. In this respect PCR-ribotyping can be thought of as a form of PCR-RFLP utilizing the rRNA gene. Although not as discriminatory as PFGE and possibly RAPD typing, PCR-ribotyping has the advantage of producing stable, easily detectable amplification patterns in a rapid manner and has the potential to be widely useful in molecular epidemiology (Kostman et al. 1992).

Insertion sequence (IS)—PCR takes advantage of the insertion sequence elements that have been discovered in several bacteria. These elements are mobile genetic units that can vary in physical location within the chromosome of different isolates and therefore make an appealing target for molecular typing. Primers can be designed to flank the ends of the IS element and face outward to amplify the intervening space. If two IS elements are close enough, then the PCR operates efficiently and a DNA fragment is amplified. This approach has been taken with organisms such as *E. coli* (Thompson et al. 1998), *Mycobacterium* (Devallois and Rastogi 1997), and *S. aureus* (Deplano et al. 1997).

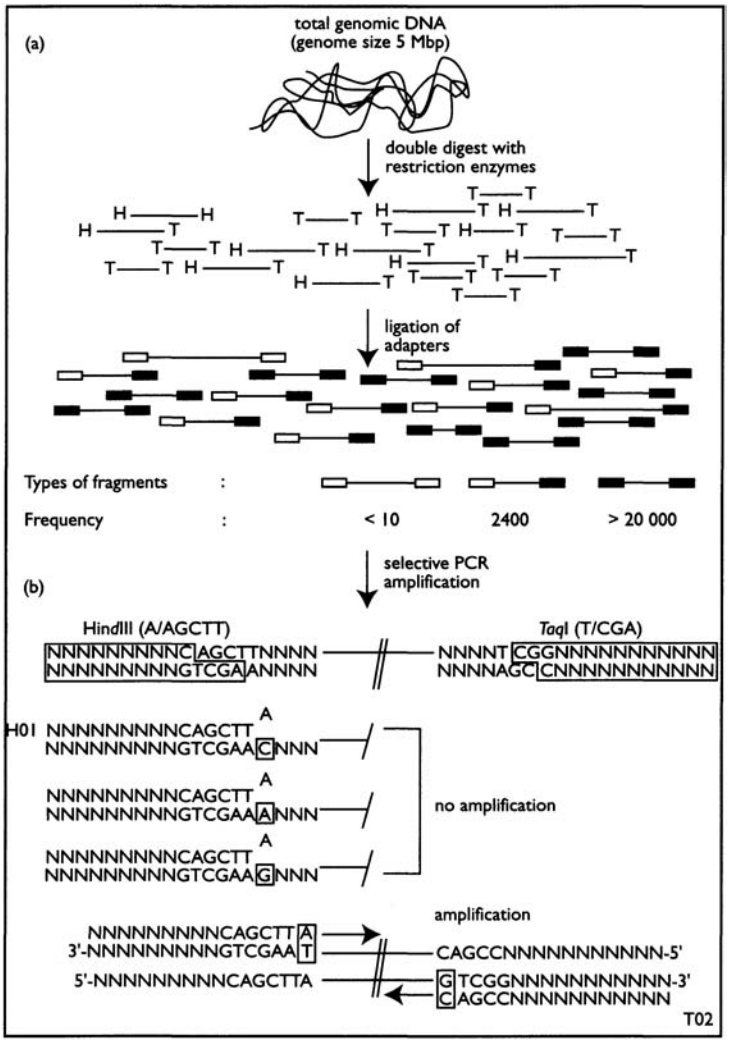
Amplified restriction fragment length polymorphism (AFLP) is a DNA fingerprinting technique involving RFLP and PCR (Janssen et al. 1996; Vos et al. 1995) (see Figure 8.1). Whole genomic DNA is isolated, then digested with a specific combination of two restriction endonucleases (REs). The first must be a frequently cutting RE such as H *MseI* , HindIII, or *Taq*I that recognizes a specific sequence of 4 bases. This generates numerous small DNA fragments that are in the optimal size range for amplification and separation. A second infrequent or rare cutting RE is also incorporated into the digestion to further reduce the number of potential amplicons, because the system is set up to amplify only those fragments having a different restriction site at each end. This latter feat is accomplished through the use of specially designed double-stranded (ds) adapter sequences that will ligate to the overhanging ends of the restricted fragments and then be amplified by primers that recognize the adapter sequence. Two adapters are required, one specific for each of the restriction sites generated. Adapters consist of a core sequence of 11 to 14 homologous ds bases that are arbitrarily selected, followed by the specific addition of bases at the 5’ end to provide an overhang suitable for ligation to one of the restriction sites (i.e., an enzyme specific sequence). A convenient omission that does not affect the results is that adapters are not phosphorylated, and thus only one strand of adapter will ligate to the restricted fragment. Ligation will, however, occur in several different ways to the various combinations of restriction fragments generated by the two REs, but only those that ligate in the proper orientation and in the desired combination will be amplified, because of the design of the primer set. Each single-stranded primer consists of a core sequence that is homologous to the majority of bases in the core sequence of the respective adapter molecule, plus the 5’ overhanging enzyme specific sequence. In addition, 1 to 3 arbitrarily selected nucleotides are added to the 3’ end of the primer. Each addition of a selective base to the AFLP primer reduces the number of amplicons approximately 4-fold and results in a subset of the original fingerprint. Usually one primer (usually the rare cutter) is radioactively labeled, and primers are annealed at high temperatures to ensure stringency. In this way, the system will amplify only the specific subset of properly ligated adapter-fragment hybrids that are homologous for the selective bases added to the 3’ end of the primer. This addition of selective bases provides a further specificity to the primer and in so doing reduces again the number of potential amplicons. This technique still results in a DNA fingerprint pattern of approximately 50–100 fragments of varying sizes and thus most typically requires a denaturing polyacrylamide (sequencing) gel for adequate resolution. Gels are visualized by autoradiography or phosphoimaging; DNA analysis software is recommended to properly interpret the likelihood of two strains being related because of the quantity of bands.

The AFLP technique has been used to study the intraspecific genetic variability within closely related species of *Vibrio vulnificus* (Covadonga et al. 1997), *Staphylococcus epidermidis* (Sloos et al. 1998), *Helicobacter pylori* (Gibson et al. 1998), and *Salmonella* (Aarts et al. 1998). It has also been applied to the determination of molecular markers in cattle (Ajmone-Marsan et al. 1997) and barley (Becker et al. 1995). A recent review on the subject can be found in the paper by Blears et al. (1998).

Identification with Primers and Probes

Disposable commercial kits may at first appear expensive and wasteful, but this aspect is more than offset by their relative cost savings and convenience. They have proven to be very economical because several samples can usually be processed at a

Figure 8.1 Overall scheme of the AFLP technique (Janssen et al. 1996).



(a) Total cellular DNA is digested with two restriction enzymes, Msel, Hindlll (H) or *Taql* (T), which have a 6 and 4 bp recognition sequence, respectively.This is followed by ligation of adapters to both ends of the restriction fragments. Adapters are restriction half-site specific and are indicated by boxes (white and black for H and T half-sites, respectively); the estimated frequency (number of fragments per genome) for each type of restriction fragment is based on the theoretical number of cleavage sites for 6 bp cutters (every 4096 bp) and 4 bp cutters (every 256 bp), assuming random base distribution and a genome size of 5 mega (1 03) bp.
(b) In selective amplification of restriction fragments, the adapter sequences serve as binding sites for the PCR primers, which contain selective bases at their 3'-end (primer sequences consist of the complementary sequence of the corresponding adapters and include the restriction site sequence). Under stringent PCR conditions, only perfectly matched primers will be elongated, resulting in amplification.As an example, selective amplification is depicted with Hindlll-primer HO I and Tag/-primer T02. Because only the Hind/l/-primer is radioactively labeled, amplified Tuql-Tug/ fragments will not be visualized by autoradiography. For clarity, only essential sequences are shown.
From Janssen et al. 1996. ***Microbiology*** 142, Pt 7. (Reprinted with permission of Society of General Microbiology.)

substantially reduced per unit cost compared to more traditional methods, which require skilled-labor hours and dedicated equipment. Microbial identification with commercially available primers and probes is very appealing because of its specificity and simplicity. Commercial kits are generally quite rapid and have been optimized to reduce hands-on time and cost per sample.

Qualicon Inc., a subsidiary of DuPont (United States) has developed PCR-based detection and identification systems employing primers targeting an undefined region of the bacterial chromosome that can be used for diagnostic screening as well as culture confirmation. Although originally designed for the detection of microorganisms in enriched food samples following a lysing procedure that releases DNA into the media, the system performs equally well for the identification of colonies on plates. It is simply a matter of substituting the enriched food sample broth with a colony suspension and lysing the whole cells directly. Currently, the BAX® system is available for *Salmonella, E. coli* O157:H7, and *Listeria* spp., including *L.*

monocytogenes. Results are obtained within 26 hours at a cost of slightly more than $1 Cdn per sample from as little as 10^5–10^6 CFU/mL; correlation is quite high with conventional assays, even in the presence of background DNA (Bailey 1998; Stewart and Gendel 1998).

Gen-Probe Inc. (United States) has developed a simple DNA probe-based chemiluminescent detection assay that targets a highly conserved region of rRNA specific to a particular organism. A rapid 3-step process incorporates sample preparation, hybridization, selection, and detection all in a single reaction tube. A recent upgrade to the system has integrated a transcription mediated amplification (TMA) step into the single tube assay prior to the probebased identification step, to enhance sensitivity for small microbial loads. Because TMA is an isothermal process, no thermocycler is required, and the assay can be carried out in a heating block or water bath (Hill 1996). Results are interpreted with a commercially available luminometer that has been optimized for the system. Identification can be performed from clinical specimens as well as pure cultures. The system has been used extensively for the detection and identification of sexually transmitted disease organisms (Chomvarin et al. 1997; Crotchfelt et al. 1998) and mycobacterium (Lang et al. 1998; Martin-Casabona et al. 1997), but kits are also available for food-borne pathogens such as *S. aureus* (Freney et al. 1993), *Listeria* spp. (Monfort et al. 1998), *Lactococcus* spp. (Teixeira et al. 1996), and *Campylobacter* spp. (Product/Technical literature/support 1999a), as well as other pathogens such as *Streptococcus* spp. (Mundy et al. 1998), *Enterococcus* spp., and *Haemophilus influenzae* (Daly et al. 1991) (Product/Technical literature/support 1999a). Results are obtained the same day (in most cases within the hour) at a cost of $5–8 Cdn per identification, depending on reagents and equipment needed for the particular organism, from as little as 10^5 CFU/mL, a 1 mm single colony, or one copy of DNA with a very high specificity (Freney et al. 1993; Hill 1996).

The Probelia™ PCR system developed at Institut Pasteur and distributed solely through Biocontrol Systems, Inc. (United States), is a recent addition to the PCR kits available for testing food pathogens. Probelia directly detects highly specific and patented genetic sequences for food-borne pathogens, from limited starting material, with next-day results. Routine screening and confirmation of food-borne pathogens is performed in a convenient microplate format. Kits are available for *Salmonella spp., L. monocytogenes, E. coli* O157:H7, *Campylobacter spp.,* and *Clostridium botulinum* (Product/Technical literature/support 1999e).

MicroSeq™ from Perkin-Elmer Applied Biosystems is a DNA sequencing-based kit. The kit consists of 6 forward and 6 reverse DNA primers based on the conserved regions of the 16S rRNA gene used in a one-step multiplex PCR reaction mixture. The amplified product is then added to 12 one-step sequencing mixes (one for each primer) and subjected to cycle sequencing with dye terminators. The 12 primers are sufficient to span the entire 16S rRNA gene in both forward and reverse directions with good overlap. The 12 separate amplified sequencing reactions are loaded onto a sequencing gel and run to completion. The choice of sequencing apparatus depends on the volume of samples and throughput desired, as well as economies; a Perkin-Elmer 377 DNA sequencer costs about $164,000 Cdn. It is a gel-based system that is said to give better read lengths than the alternatives do. It can be equipped to handle 36, 64, or 96 samples, depending on the upgrade package, and takes between 4 and 7 hours per run (a slower run means better resolution of bases). The Perkin-Elmer 310 ($80,300 Cdn), based on capillary electrophoresis (CE), does not require a gel to be run. Run times are faster than the 377 model, 2.5–3 hours, but it can handle only one sample at a time. However, 48 or 96 samples can be set up, depending on the loading tray, and then ignored until all samples are done; of course, this would take several days. Perkin-Elmer now has a 96-sample CE-based system, the 3700, that will run all samples at once, but the cost is $430,000 Cdn (Product/Technical literature/support 1999d). The results of sequencing are compared to the microbial identification and analysis software database library of microorganisms to determine the similarity percentage of the unknown samples to the extensive samples in the database. This particular software can identify more than 1000 species on the basis of the sequencing results. Because this is a PCR-based kit, assays can be performed with a minute amount of starting material and contaminants easily identified separately from the main isolate, on the basis of electropherograms.

Two options for the kit will work with any system. The full version provides a complete sequence of about 1,500 bp at a cost of $850 Cnd for 10 samples or about $85 a sample. Alternatively, the 500 bp read kit, for 50 samples, $17 per sample. Because most mutations occur within the first 500 bp, this should be sufficient for the identification of most organisms. The system has been used for the identification of unusual aerobic pathogenic gram-negative bacilli (Tang et al. 1998).

CHEMOTAXONOMY

As an alternative to direct nucleic acid fingerprinting, there are numerous biochemical techniques. Chemotaxonomy involves the application of chemical and physical manipulations to the analysis of the chemical composition of whole bacterial cells or their cellular components to arrive at some identification or taxonomic positioning. Even with accelerated advances in technology that have allowed for miniaturization and automation of analytical equipment, the bacterial growth period and chemical derivations prior to analysis still remain the ultimate limiting factor with respect to rapid analysis. Thus, methods amenable or adaptable to whole-cell techniques requiring only minute quantities of sample and/or in situ chemical derivations are of particular interest. Methods such as mass spectrometry (MS) and gas or liquid chromatography (GC or LC) are based

mostly on ionization of sample molecules, which occurs when the molecule is allowed to absorb high energy from incoming photons, causing photo-excitation and the complete removal of an electron from its molecular orbit. The ions generated can then be directed, filtered, and detected to present a chemical finger-print of the original sample. Other methods, such as infrared spectroscopy, induce molecular vibrations, rotations, or some other change in the energy state of the sample molecules that promotes electrons to higher orbitals. This change in energy state can then be measured and recorded.

Several chemotaxonomic techniques have been combined into so-called "hyphenated methods," such as GC-MS, LC-MS, Py-GC-MS, or LC-FTIR, to further enhance their discriminatory power. One such approach has been the application of pyrolysis (Py) to gas chromatography (GC) for the in situ thermal hydrolysis and methylation (THM) of whole-cell bacterial fatty acid methyl esters (FAMEs). This has also been applied to mass spectronomy, without a chromatographic step (Basile et al. 1998a; 1998b). The following automated, rapid instrumental methods are of particular value.

Mass Spectrometry and Pyrolysis

Mass spectrometry (MS), which has been developing since the early 1900s, involves the characterization of components based on their chemical structure. However, only since the 1990s has MS developed into a technique accessible to the average researcher, because of the falling cost of dedicated, automated equipment that works intimately with computer control and analysis software. MS provides a rapid alternative to traditional nucleic acid fingerprinting techniques, delving further into the biochemical makeup of the cell with far more precision. Multiple samples can be efficiently and reproducibly processed with much less personal attention. In addition, the development of sophisticated multivariate statistics and artificial neural networks (ANNs) has allowed MS to become a practical and efficient method for the analysis of microorganisms.

There are three basic stages to mass spectral analysis once sample introduction into the vacuum environment has been achieved: (1) creation of charged particles by using some ionization source; (2) separation of ions in space based on their mass to charge ratio (m/z or m/e) by means of some mass selection analyzer, and an acceleration technique to drive them toward a detector (mass analyzers are just ion filters that separate different parts of the electromagnetic spectrum by absorbing or reflecting certain wavelengths and transmitting others); and (3) measuring the quantity of ions at each m/z ratio by using some ion detector and recording the peaks.

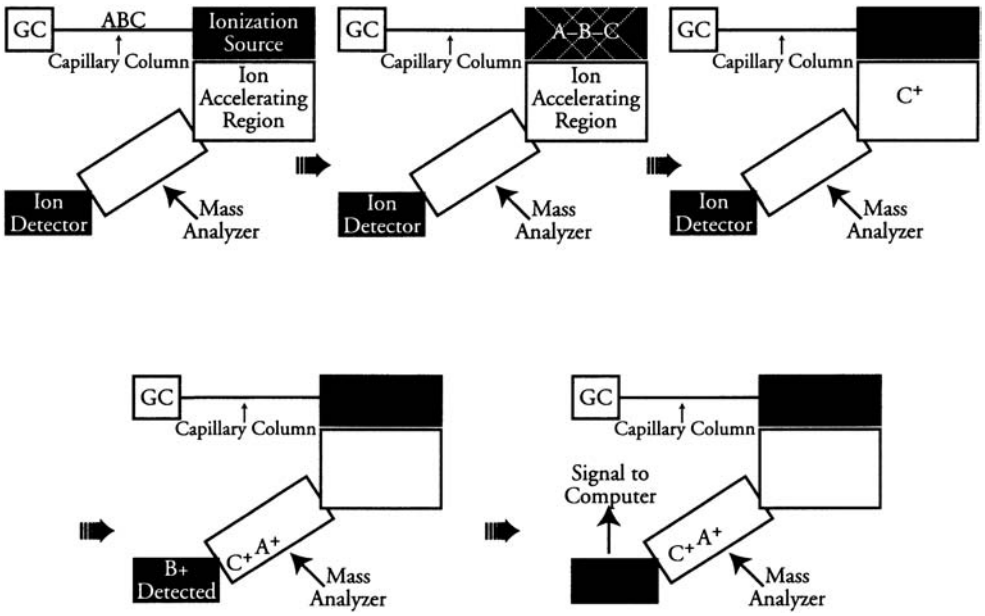
MS works by applying an electric or magnetic field to charged particles (ions) in a vacuum, directing, filtering, and then detecting them. A compound must first be introduced to the vacuum environment in a volatile (gas-phase) form and be charged or ionized before it can be analyzed by MS. Ionization has generally been used to refer to the creation of gas-phase ions from gaseous or heat-volatile analytes, as well as the creation of ions from nonvolatile or thermally labile compounds. However, the latter approach is more accurately referred to as desorption or desolvation, as it involves releasing ions from the surface of solid materials and involves separate and varied techniques. Mass spectrometers measure the m/z of ionized particles and generate a graph of peaks. Peaks are separated or resolved on the basis of the size of the m/z ratio, the height of the peak representing the abundance of a particular m/z ratio. Resolution or resolving power refers to the ability of the technique to distinguish among the various m/z ratios.

Advances in ionization-desorption methods such as electrospray ionization (ESI) and matrix assisted laser desorption-ionization (MALDI), combined with separation techniques such as time of flight (TOF) and the quadruple ion trap, have allowed for the manufacture of benchtop MS models that rival the sensitivity, specificity, and speed of the larger, more expensive, higher end magnetic sector instruments. Biomedical analysis and characterization of large, sophisticated biomolecules such as DNA, peptides, and proteins can be studied at low cost with these models, compared to the higher end instruments.

MS may be used as a stand-alone analyzer or as an add-on detector system to augment other techniques. It is widely used as an add-on for gas chromatography (GC) or liquid chromatography (LC) in pharmacological, forensic, and environmental analysis. In this way, chemically derived products can be purified and analyzed with much higher sensitivity than with GC or LC alone.

LC-MS combines high performance liquid chromatography (HPLC) with, most typically, ESI-MS. The sample coming out of the column is sprayed into the vacuum environment of the MS, and ionization occurs. The resulting ions are then guided along to the detector, which generates a peak. GC-MS (see Figure 8.2) combines gas chromatography with, most typically, electron impact (EI) ionization, in which the sample leaving the GC is bombarded directly with an electron beam in the MS environment. MS takes place in a high vacuum, and the carrier gases from the GC must first be separated from the molecules before they enter the MS chamber. GC-MS can be performed equally well with chemical derivations or analytical pyrolysates. The latter involves the use of flame ionization prior to the sample entering the chamber. Quadruple mass filters and ion traps serve equally well as GC or LC detectors.

Figure 8.2 Schematic of GC-MS.



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Pyrolysis MS

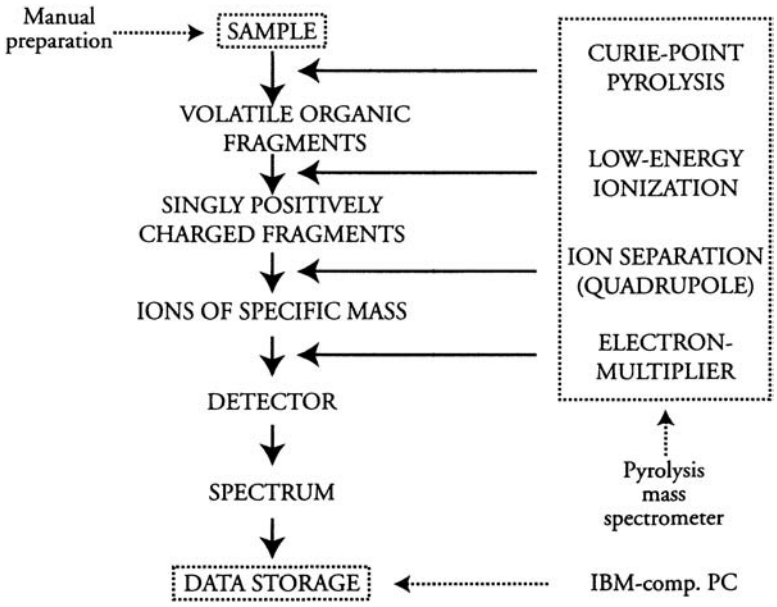
Nonvolatile biological materials such as whole-cell microorganisms can be made accessible to the high sensitivity of mass spectral analysis by means of the pyrolysis technique. Pyrolysis, a chemical process, involves the thermal degradation of nonvolatile, organic matter. Samples are rapidly heated in a vacuum (called the pyrolyser or pyrolysis chamber) to ultra-high temperatures to thermally degrade (or vaporize) a complex material at its weakest points into smaller volatile fragments called pyrolysates (Irwin 1982). The pyrolysates then leak into the ionization chamber (also under vacuum), at low concentrations, where various means have been employed to create the gas-phase ions necessary for analysis (called the analyte). Pyrolysis can be applied equally well to solid or liquid samples, chemical derivatives, or whole cells. Although pyrolysis has a long history of use with GC standalone systems (Leathard and Shurlock 1970), it was the combination of pyrolysis with mass spectronomy (Py-MS) (see Figure 8.3) that allowed MS to be adapted for use with whole cells as well as isolated compounds and provided a general picture of the chemical composition and structure of the sample analyzed.

Many pyrolysis techniques, such as infrared laser, low-voltage electron impact, field ionization, and pyrolysis field desorption have been investigated. However, Curie-point pyrolysis has been shown over the years to be the best and most reproducible technique for pyrolysis of microorganisms. Results may be obtained in less than 2 minutes starting with a single colony (1–10 µg) from a pure culture (Freeman et al. 1995a; Goodfellow et al. 1997). A complete setup is about $104,000, but the cost per sample is quite low—about $2.40 US. A run may consist of 150 to 300 samples, depending on the holder, but samples are usually run in triplicate; thus, one can gather data on 50 to 100 organisms per run at a cost of between $360 and 720 US (Product/Technical literature/support 1999f).

A complication with interpreting mass spectral data stems from the complex pattern of broad and overlapping peaks, which make direct visual analysis impractical, if not impossible. Thus, it is necessary to employ highly sophisticated statistical and mathematical methods that have been developed for the purpose of sorting out this chemical data. Known as chemometrics, the workers in this field attempt to handle hyperspectral data by limiting analysis to a defined region or to combinations of regions known as "spectral windows" and applying sophisticated mathematical filtering techniques to reduce the complex data. For MS, the useful range of m/z ratio for differentiating most microbiological species has been found to be between 51 and 200 atomic mass units (amu). This is the range that has been focused on the most in analysis of microorganisms for the purpose of identification and typing. Fortunately, this process can be handled by statistical analysis packages that may be available as computer software accompanying currently marketed devices. This computerization offers the distinct advantage of being able to rapidly access huge databases of stored spectral patterns that can be customized and expanded. Py-MS is thus appealing because of its total automation, involving the researcher only to grow and prepare minute amounts of sample. Analyses may also be performed directly from freeze-dried samples (DeLuca et al. 1990).

Of specific interest in the pharmaceutical industry is the screening of microorganisms for the production of biologically active metabolites. It is also important to be able to rapidly detect low levels of microbial contamination in the production process at an early stage, and to monitor with high sensitivity the effect of drugs on the cell. However, successful evaluation

Figure 8.3 Flow diagram of the main steps in pyrolysis mass spectrometry.



From Goodacre et al. 1996. *Current Opinion in Biotechnology, 7.* (Reprinted with permission of Dr. Roy Goodacre.)

seems dependent on the appropriate use of chemometrics; some species have been correctly classified only when a specific algorithm was used (Goodacre et al. 1998b; Holt et al. 1995; Lefier et al. 1997). Most recently multivariate statistical methods such as principal-component analysis (PCA) (Jolliffe 1986) combined with the ANN approach have been highly successful in discriminating bacterial species from convoluted mass data. ANNs are becoming increasingly useful with techniques that require pattern recognition. As a "supervised learning approach," ANNs can be trained, with representative spectra, to recognize novel patterns and provide rapid and accurate bacterial identification (Goodacre et al. 1996a).

Py-MS has been applied to the in situ THM of whole-cell bacterial FAMEs without a chromatographic step (Basile et al. 1998b; DeLuca et al. 1990), by using an infrared pyrolyser with a quadrupole ion-trap mass spectrometer (Basile et al. 1994). Lipids (or fatty acids) are unique biochemical markers for the purpose of identification and classification in bacteria. Air is used as the carrier gas, and pyrolysis occurs in the presence of trimethylphenylammonium hydroxide (TMAH), whereby lipids are methylated rapidly within the vacuum chamber. This procedure saves the lengthy step of preparing the chemical derivative and running it through a column. Ionization occurs primarily by electron ionization, but a secondary chemical ionization effect is also present. The whole process can be reduced from >1 hour, with a traditional GC or the MIDI microbial identification system (MIS) (see below) approach, to about 1 minute. The identification of nucleic acids in whole cells has also been accomplished by in situ methylation to increase the volatility of nitrogen bases by having TMAH present (Abbas-Hawks et al. 1996).

Py-MS has been used for taxonomic classification, microbial identification, and chemical typing of bacteria down to the intraspecies level, as well as for purification of chemically derived components by combining it with GC or LC. It has also been applied to the analysis of bacterial DNA (Mathers et al. 1997) as well as the detection of differences in same-length oligonucleotides that vary only in their base sequence (Freeman et al. 1994b). Characterization of PCR products has also been accomplished with ESI—Fourier transform ion cyclotron resonance (FTICR) MS (Muddiman et al. 1996). For bacteria, microbial "typing" has been the main application. Some examples include: (1) investigation into changes in cell composition during exposure to antimicrobial agents (Magee et al. 1997); (2) in-source pyrolysis of polysaccharides by using desorption chemical ionization (DCI) and Curie-point Py-GC-MS (Lomax et al. 1991); (3) differentiating *Staphylococcus* and *Streptococcus* spp. on the basis of whole-cell pyrolysis vs. pyrolysis of DNA extracts, showing that Py-MS can detect genetic as well as phenotypic differences (Freeman et al. 1997; Mathers et al. 1997); (4) investigation into the nosocomial spread of enterococcal infection and showing the inadequacy of existing enterococcal decontamination procedures in hospitals (Freeman et al. 1994a); (5) interstrain comparison of *S. aureus* associated with nosocomial infections (Sisson et al. 1998); (6) discrimination between MRSA and non-MRSA, to demonstrate the power of Py-MS as a method for antibiotic susceptibility testing (Goodacre et al. 1998a); (7) analysis of an outbreak strain of *Listeria monocytogenes* serovar 4b (Freeman et al. 1991); (8) investigation of an outbreak of food poisoning from *Bacillus* spp. and *Clostridium perfringens* (Sisson et al. 1992); (9) characterization and identification of *Bacillus* spp. (Shute et al. 1984); (10) typing of *Salmonella* spp. and *Streptococcus pyrogenes* (Freeman et al. 1990); (11) correlating Py-MS with outer membrane protein profiling (cell surface antigens) in

Clostridium difficile to evaluate its potential as an alternative to serotyping (Ogunsola et al. 1995); and (12) rapid differentiation and identification of urinary tract infection bacteria and yeast (Goodacre et al. 1998b; Timmins et al. 1998).

Reproducibility

The lack of short-term interlaboratory reproducibility of Py-MS has been attributed mainly to the use of various pyrolysis techniques and devices (Shute et al. 1988; Windig et al. 1979). Growth and/or subculturing practices applied to cultures must be strictly standardized (Ward et al. 1994). In addition, the destructive nature of pyrolysis must be controlled to avoid secondary reactions. This can be accomplished by controlling temperature rises and heating times and rapidly moving the pyrolysate along the chamber (Schulten and Lattimer 1984; Windig et al. 1979). Variation in sample size plays only a small role in terms of variability and can be minimized by normalization techniques. Failure in long-term reproducibility, on the other hand, is mostly attributed to mass spectral drift, which results from contamination and aging of the instrument (Goodacre and Kell 1996a; Windig et al. 1979).

Because factor analysis and other pattern recognition techniques must be used to interpret the complex mass spectra, there is an element of subjectivity introduced by the analyzer, because of not only the methods chosen but also the interpretation of results. However, the use of standardized computer software reduces this effect. Finally, the resulting mass spectral patterns contain several peaks that have not been associated with known biochemical markers (Heller et al. 1988), and many low-mass species are likely to be pyrolysis fragments of larger molecules and not distinct chemical derivatives. However, these effects can be minimized by focusing on particular known regions.

Consequently, with the limitations in reproducibility of Py-MS, it is restricted in its applications. Py-MS is thus left in the familiar category of a technique that should be applied only to "operational fingerprinting." The data generated are only reliably interpreted when test strains are analyzed along with reference or type strains in the same analytical run, or when the technique is used in combination with other typing methods and not applied to long-term taxonomic positioning. However, recent improvements in the application of statistical techniques such as artificial neural network analysis (Chun et al. 1997; Freeman et al. 1995b; Goodacre and Kell 1996a) may provide solutions to the dependable use of chemotaxonomic techniques and may well be the future of routine identification, classification, and typing of microorganisms.

The Web page http://gepasi.dbs.aber.ac.uk/roy/pymshome.htm (Goodacre 1996b) has information on ionization methods, mass selection analysis, and ion detection in Py-MS, as well as links to several sites with background information. Further reading, including detailed protocols on Curie-point Py-MS as a tool for clinical microbiology and applications to biotechnology, can be found in reviews by Freeman et al. (1995a), Goodacre and Kell (1996b), and Goodfellow et al. (1997). Information on deconvolution of mass spectral data for Py-MS and application of artificial neural networks can be found in reports by Goodacre et al. (1996a) and Kenyon et al. (1997).

Fourier Transform Infrared Spectroscopy

The potential of using infrared (IR) absorption spectrums acquired from IR spectrometers for bacterial analysis dates back to the mid-1950s. However, only since the early 1990s has Fourier transform infrared spectroscopy (FTIR) been developed into an efficient method for the bio logical characterization of intact bacteria by using dedicated, automated equipment. It has been touted as being at the forefront of new potential techniques that can be used for the rapid screening of microorganisms. Improvements in FTIR spectrometers that provide high signal-to-noise ratios and precision in absorbance and wave number measurements, as well as new accessories for sampling, have contributed significantly to extending the application of infrared technology and have sparked a resurgence in its use for the characterization of microorganisms (Holt et al. 1995). These advances have been further aided by the development of huge libraries of spectral patterns that can be readily compared to unknown profiles by means of computer-assisted search algorithms.

FTIR as a spectroscopic technique can be used to qualitatively identify bacterial and environmental samples, as well as quantitatively measure the concentration of a particular constituent component. It measures the vibrational characteristics of functional groups and polar bonds when excited by electromagnetic radiation (light) in the infrared spectral region. The infrared beam is made up of bands of light, each with a distinct wavelength. When a sample is placed in the path of the beam, particular functional groups absorb the energy from particular wavelengths. The energy transferred from the particular IR band to a particular molecular species sends its electrons to a higher energy level, inducing the molecular species to vibrate with a unique frequency that is structure dependent. The transmitted signal reaching the sensor is thus attenuated by the loss of particular wavelengths. The frequency at which the sample absorbs the radiation and the intensity of the absorption can be recorded and an absorption spectrum created.

Because chemical functional groups are known to absorb light at a specific frequency (i.e., at a particular wavelength number or within a narrow range), every known compound expresses a unique vibrational spectrum, and a unique absorption profile will be generated when all structures making up the complete sample are considered. FTIR results can thus be seen as

bio-chemical fingerprints and can be used as a reliable identification tool for unknown samples by comparing the spectral patterns generated to a stored library within a computer database. This technique is most widely used in the field of forensic science and in the pharmaceutical industry, where accurate quantitative and qualitative data are essential. It has been shown to be a rapid and highly reproducible method when correctly applied (Helm et al. 1991a). After the initial investment in equipment, it is also very economical on a per sample basis, because several samples can be set up and run at one time.

The discriminatory power of FTIR is quite high; it can differentiate microorganisms down to the subspecies and, in most cases, the strain level. Spectral patterns are based on the vibrational characteristics of cell constituents such as the nucleotide sequences that make up DNA and RNA, the amino acid conformations that result from translation to protein, the products of protein expression, and the phospholipids, polysaccharides, and fatty acids that make up structural membranes and cell wall components. The generated infrared spectrum is thus a result of phenotypic expressions as well as genetic structures, making FTIR a complete assay of total cell composition.

As with chemotaxonomic methods, there is a range that has been shown to be useful for the discrimination of microbial species. Naumann et al. (1991; Helm et al. 1991b) have shown that the mid-IR range, approximately 4,000–200 reciprocal wavelengths or wave numbers (per centimeter), is sufficient for intraspecies discrimination. For this reason, FTIR is most useful in the identification and quantification of organic and organometallic molecules. Reliable microbial identification down to the species or even strain level can be accomplished within this range. Further, the region associated with the bacterial cell wall (1,200–900/cm) has been shown to be the most useful in distinguishing among microorganisms, because it contains the most variability when comparing spectral fingerprints.

FTIR is a rapid technique for microbial differentiation, classification, identification, and largescale screening at the subspecies level (Helm et al. 1991b; Naumann et al. 1991). It can be used reliably for strain classification, rapid identification of clinical isolates (Helm et al. 1991b), and tracking of outbreak strains, as well as for detecting genetic drift during the maintenance of stock cultures or reference strains. Unlike other chemotaxonomic techniques, FTIR is nondestructive and does not have numerous variations for sample introduction and processing. It is most suitable for the characterization of structural features and biological molecules of complex materials, but it can also be used for monitoring the physical state of biochemical compounds (i.e., the structural changes that may occur as a result of environmental pressures and experimental conditions) (Sockalingum et al. 1997; Zeroual et al. 1994). Bacteria with different biochemical and genetic structures will have different FTIR spectra; thus, unique molecular fingerprints can be generated and easily distinguished on the basis of absorbance patterns. An intact or whole-cell approach can be used to generate IR fingerprints, thus avoiding complicated and time-consuming chemical or component isolations. Proteins, membranes, cell wall, and nucleic acids all get analyzed. The applications are easily applicable to all microorganisms such as viruses, fungi, yeasts, and amoebae, as well as mammalian and plant cells, even though the method was initially developed for bacteria (Naumann et al. 1991). It is a simple, uniform procedure that is applicable to all bacteria, and many samples can be included in the same experimental run (limited only by size of sample holder). The method has been useful in determining the extent of bacterial colonization in general (i.e., how many colonizing strains and where located), cross-infections in an environment, and the tracking of genetic changes in microorganisms over time. It is capable of detecting microorganisms directly from food, clinical, or environmental samples and of distinguishing among genera, species, and subspecies for the purpose of identification and grouping of various strains (i.e., typing) into clusters and subclusters (e.g., subdividing biotypes of the same species). It has been used for the identification and typing of various microorganisms such as *Listeria* spp. (Holt et al. 1995; Lefier et al. 1997), *Bacillus* spp. (Lin et al. 1998), *Pseudomonas aeruginosa* (Sockalingum et al. 1997), *Streptococcus and Enterococcus* spp. (Goodacre et al. 1996b), *Staphylococcus, Streptococcus, Clostridium, Legionella, E. coli, Aeromonas, Pseudomonas, Yersinia,* and various Enterobacteriaceae (e.g., *Proteus, Providencia, Serratia, Klebsiella, Hafnia, Edwardsiella, Enterobacter, Citrobacter*) (Helm et al. 1991a, 1991b). The method has been shown to be highly reproducible when tested under various conditions and replicate sampling (Helm et al. 1991a), and characteristic peaks are found not to be affected by the composition of growth media (Lin et al. 1998). Late exponential phase cells from confluent colonies in the third quadrant of an agar surface have been shown to be the most reproducible (Miller 1982). Results may be obtained in less than 1 hour at a cost of about $2 per test, starting with as little as 10 μg of pure culture (Helm et al. 1991b; Naumann et al. 1991). Even microcolony spots of approximately 40 μm in diameter or $<10^4$ bacterial cells are adequate (Naumann et al. 1991). The initial investment in a completely automated system would be between $37,000 and 45,000 US.

The Web page http://gepasi.dbs.aber.ac.uk/roy/ftir/ftirhome.htm (Goodacre 1997) has more information on FTIR as well as links to several sites with background information. Also see http://www.elchem.kaist.ac.kr/vt/chem-ed/spec/vib/ir-bands.htm (Tissue 1996) for a listing of functional groups and their respective wave numbers or ranges. Other general articles dealing with protocols, functional groups and FTIR applications to biotechnology are those of Franck et al. (1998), Helm et al. (1991a, 1991b), Holt et al. (1995), and Lin et al. (1998).

Gas Chromatography and the Microbial Identification System

Gas chromatography (GC) involves the separation of volatile organic compounds such as FAMEs. Fatty acids (FA) are innately nonvolatile but are made so through methylation, which creates methyl esters. A methyl group is attached to the carboxyl group of the FA after esterifi cation to make it volatile; otherwise it could not be detected. Once vaporized into gas phase molecules, the FA can be distributed onto the column by being carried along with the inert carrier gas, within a flowing mobile phase, such as helium, argon, nitrogen, or hydrogen as it passes over a stationary phase.

The choice of the mobile phase depends on the application. For example, hydrogen is generally used as the carrier gas for analysis of FAMEs because neither helium nor nitrogen properly separates certain FAs. GC can be gas-solid chromatography (GSC) or gas-liquid chromatography (GLC), depending on the type of stationary phase column used. The stationary phase coats the walls of the column or packing material. GSC contains high-surface-area inorganic or polymer packing, whereas GLC contains a liquid stationary phase on a solid support. FA analysis is typically GLC. In addition, GC columns can be of two types, capillary or packed; the choice depends on the application. Capillary columns are much thinner and provide much higher separation efficiency than packed columns; however, they can handle only small amounts of sample and are therefore more easily overloaded. They also require specialized pressure control devices to maintain a high pressure. Applications such as FA analysis require capillary columns.

The sample is typically sprayed onto the column (similar to ESI in mass spectronomy) through a heated injector maintained at a temperature above the boiling point of the least volatile species in the mixture (approximately 250°C for FA). The vaporized gas phase molecules are swept along with the flowing inert carrier gas through the column to the detector, where they are ionized by various means and the resulting ions detected as a peak similar to that already described for MS. Separation is based on retention times and controlled by the temperature of the column. A wide range of components can be efficiently separated by slowly increasing the oven temperature over time (e.g., from 170 to 270°C for FAs). Retention is based on the length of the molecule and the extent of branching, long-chain, heavily branched molecules being retained on the column longer than shorter, minimally branched ones. With FAs, for example, retention times are converted by a mathematical formula inherent in the software to an equivalent chain length (ECL) based on runs with calibration standards of known chain lengths. The areas under the peaks are calculated to arrive at the concentration of ions, at a particular peak, given as a percentage of the total molecular ions. When compared with the appropriate computer database, a similarity index (SI) can be generated to tentatively identify the sample (SIs range between 0 and 1; 0=no match, 1 = perfect match). The database contains a peak naming table, which is a series of ECLs associated with particular retention times and an FA name. It is important to realize that the chemically distinct profiles for the FAs in the database have been created on the basis of a particular set of parameters. It is therefore crucial to follow the exact protocol for the database to properly interpret the results.

With GC, unknown isolates may be identified to the genus, species, and subspecies level, depending on the extent of data in the database and the associated SI. The relative abundances of compounds in the sample are also presented. Commercial databases are available for a wide range of microorganisms, but they must be purchased separately as individual databases (aerobes, anaerobe, yeast-fungal-actinomycete; see MIDI [1999]) for a complete updated list of organisms in this company's databases).

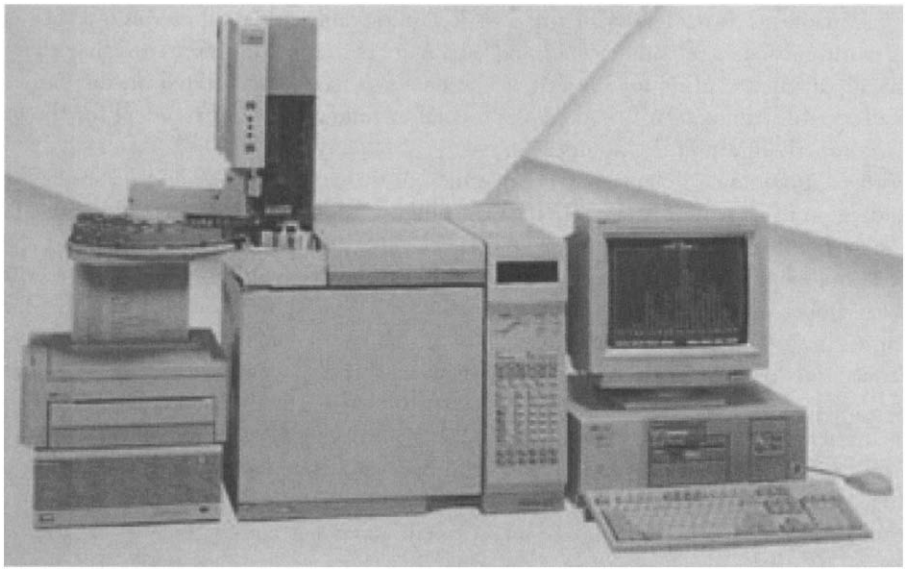
Detection occurs upon exit from the column and is actually a combination of ionization and sensor devices. This process, like mass spectrometry, can be destructive as well as nondestructive when further spectroscopic analysis is required. It is this principle that requires use of various detectors to achieve selectivity and desired sensitivity: atomic-emission, flame-ionization, and thermal conductivity detectors (AED, FID, TCD), chemiluminescence, and flame-photometric, electron capture, photoionization, and mass spectrometer detectors (FPD, ECD, PID, and MSD).

Microbial Identification System

The microbial identification system (MIS) is a GC-based FAME analysis instrument with the ability to process multiple samples. It includes a 60 minute lipid extraction-methylation from whole cells followed by a 15–20 minute chromatographic run. The resulting profiles are compared to existing computer databases, and possible matches are identified.

There is only one fully automated system dedicated to the analysis of microbial whole-cell fatty acids, the Sherlock® Microbial Identification System (MIS) (MIDI, Inc., United States). It is a GC-based system, using flame ionization, that is intimately linked to extensive computer database analysis of results (see Figure 8.4). However, one can do whole-cell fatty acid analysis by GC alone or any one of several other approaches that involve separate techniques, or combinations of them, which may offer better resolution and analysis. Results can then be compared manually to extensive commercial databases supplied with the system or available separately. MIDI software and databases can be purchased separately for pre-existing setups, so there is no need to buy the whole system.

Figure 8.4 HP GC ChemStation and Sherlock® pattern recognition software (Product/Technical literature/support 1999c)



Used with permission of MIDI, Inc., Newark, Del.

FA analysis using the MIS system has been shown to be more discriminating for microbial identification than other techniques, such as IR analysis in the fatty acid range (Lin et al. 1998). For example, Schraft et al. (1996) showed that fatty acid analysis could be successfully applied to the typing of *B. cereus,* but IR analysis in fatty acid region I did not allow differentiation from other bacteria (Lin et al. 1998). Results are obtained within 2 hours from pure cultures at a cost of approximately $1.30 US per sample, starting with approximately 4 mg dry cells to about 2.4×10^{10} cells for chemical derivations, or as little as 50 µg dry cells for in situ thermally hydrolyzed and methylated lipids (Basile et al. 1998a; Product/Technical literature/support 1999c).

Cellular fatty acid analysis is not readily accepted as a typing tool because of its poor interlaboratory reproducibility and because members of the same species are too close to discern without additional techniques; genetically similar genera may have qualitatively and quantitatively similar FA profiles, so that they are indistinguishable solely on the basis of FA analysis using GC (e.g., *Shigella* and *E. coli* are genetically quite similar, and their FA profiles are virtually indistinguishable). Thus, there is a need for add-on detectors or other forms of analysis. However, typing has been successfully attempted with *B. cepacia* from cystic fibrosis patients (Kumar et al. 1997) and *B. cereus* (Schraft et al. 1996).

ELECTROPHORETIC PROTEIN TYPING

Although whole-cell protein profiles (WCPPs) and their variations have the potential for full automation with devices such as the Biomek 2000® workstation (Beckman/Coulter Inc., United States), which can be programmed to perform any combination of repetitive laboratory techniques, this approach is currently underdeveloped.

Microbial proteins can be visualized as WCPPs or immunoblotting profiles (IPs). This technique is primarily a typing tool used for discrimination of previously identified organisms to below the species level. It is generally carried out on a semi-solid matrix such as starch or polyacrylamide. Whole proteins are extracted, purified, and separated on the semi-solid matrix by application of an electric current. The gel is then visualized through staining with a protein dye such as Coomassie blue.

The protein component of the cell reflects its biological activity, and different bacteria will have different chemical and biological makeups. Proteins fold in specific ways to carry out their unique biological or chemical function. This folding depends on the sequence of their amino acids, as translated from messenger (m)RNA, which are in turn coded for by transcription of nucleotides present in the chromosome. Thus, protein typing can be seen as an indirect assessment of the genetic makeup of the cell; by extension, whole-cell protein patterns can be considered as molecular fingerprints analogous to DNA typing. Any minor alterations in the genetic structure of the chromosome will be reflected in changes in protein folding patterns, and thus overall conformation and charge on the protein. Ultimately, changes in electrophoretic mobility affect migration rates of the protein on the gel. In this way, electrophoretic typing (ET) of protein can be used indirectly for discriminating similarities and differences between the genomes of microorganisms. It is the extreme variability of protein patterns between bacteria that allow them to be used as identification markers of a particular species. Conversely, interstrain patterns may be quite homologous, differing in only a few minor bands; i.e., within the same species, consistent banding patterns are generally

found in a certain region of the gel, but minor variations in other banding regions in the same species may allow for strain-specific typing and may be used as a molecular fingerprint analogous to DNA typing.

Protein expression is a phenotypic trait that relies heavily on environmental conditions; thus, exact standardization of culture preparations is essential for strain typing. Cells are solubilized by heating with surface active agents such as sodium dodecyl sulfate (SDS) and -mercaptoethanol, which assist in disrupting the disulfide bridges to linearize the protein; the entire components of the cell are then run on a semisolid matrix. In this method, the SDS also serves to neutralize the native charge on the proteins by leaving an adherent coating on them, making all proteins electrically equal and thus separated on the basis of size only. As SDS has a net negative charge, proteins can be separated by application of an electric current gradient and will move toward the positive electrode, identical to the separation of DNA, which also carries a net negative charge. This is generally a 2 day procedure requiring 20–40 mg (wet weight) of cells (Costas 1990).

A nondenaturing variation of electrophoretic protein typing which leaves enzyme function intact is called multi-locus enzyme electrophoresis (MLEE). This technique involves a thick, sturdy matrix, most commonly starch, in which horizontal slices can be taken for development with various substrates to assess the activity of a variety of enzymes (Murphy et al. 1990). The relative position of the enzymes on the gel will vary with small modifications in the amino acid sequences. The alterations do not affect function; rather, the change in conformation and charge of the protein affects its migration rate in an electric field. In this way, strains can be differentiated.

A more discriminating method involves the use of western blotting and probing with a labeled antibody (immunoblotting). Immunological probes look for allelic variations, an approach that has been shown to resolve differences that were not apparent when only protein typing was used (Krikler et al. 1986). Further variations include outer membrane protein profiling, in which only the outer membrane proteins are extracted, or in gram-negative organisms, lipid polysaccharide structure (LPS) profile analysis, in which the LPS layer is enzymatically extracted. The LPS is an important component of the outer membrane, as well as a key determinant of virulence and antigenic variation.

Typically, one-dimensional WCPP consists of 50 to 60 discrete bands of differing intensities, numbers that make manual interpretation difficult. Densitometers have been used to provide a degree of objective analysis with respect to WCPP, but this does not appear to be the norm. Densitometers provide a means of objectively recording qualitative and quantitative differences in banding patterns directly into a computer database for further analysis. Several software packages are readily available to perform the integrator functions necessary to arrive at migration distances and relative quantities of components. Even so, the majority of WCPP analyses have continued to be subjective interpretations and thus incorporate varying degrees of personal bias, making interlaboratory comparisons of results problematic.

CONCLUSION

Although genotypic typing methods are increasingly seen as powerful tools in molecular epidemiology, there are still issues that need to be addressed if these methods are to be routinely incorporated. Because there is no “gold standard” by which to judge a typing method (van Embden et al. 1993), careful standardization of and adherence to laboratory protocols is essential if individual methods are to be accepted with respect to classification of strains. Another hotly debated issue concerns the area of reproducibility and the lack thereof for certain techniques. Consistent reproducibility is essential if these methods are to be of value in the long-term analysis and categorizing of bacterial strains. An extremely important issue is how one interprets differences between strains when there is only a 1 to 3 band difference. Some investigators feel that a single difference in the production of an enzyme or the shift of a single band on a gel is not enough to indicate that two isolates are different; they believe that clonality should be considered as a relative concept (Arbeit 1995). The ultimate goal is the development of an ideal molecular typing method—one that is easy to perform, cost-effective, relatively rapid, amenable to statistical analysis and automation, able to type all possible strains, reproducible, and balanced between increased discriminatory power and applicability. Rapid advances are being made in typing methods based on whole-organism DNA sequencing, so we may well approach such an ideal method in the near future.

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9
Techniques for Detection of Specific Organisms and Potential for DNA-Based Technologies

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CURRENT ESTABLISHED METHODS FOR SPECIFIC ORGANISM DETECTION

Immunological Detection Systems

Immunological methods for the detection of specific analytes, including microbes, were developed by the early 1960s and have been used successfully in clinical and industrial food applications ever since. These methods rely on the specificity of the antibody or antigen reaction, of which there are many different test configurations.

Patel and Williams (1994) provided an excellent overview of the evaluation of commercial kits and instruments for the detection of food-borne bacterial pathogens and toxins. These generally offer 24-hour detection by means of either simple visual or fully automated instrumental endpoint detection methods. Some of the target pathogens, such as *Salmonellae* and *Escherichia coli,* are of concern to the pharmaceutical industry, and the conventional cultural methods are also very similar; hence, transferring the technology from one industry to another should not be difficult.

Techniques such as enzyme-linked immunosorbant assays (ELISA), latex agglutination, and gene probes have a well-proven history, and they are recognized, accepted, and used by regulatory agencies around the world. Some are specifically listed in the FDA's *Bacteriological Analytical Methods* manual (available through their Web site at www.foodHACCP.com/index3.html).

Despite the proven track record, extensive validation, and certified performance of such tests, their use for quality assurance purposes in the pharmaceutical industry has been very poor. This is perhaps a reflection of the relatively limited range of organisms that are of direct relevance to the pharmaceutical industry. For example, there are no direct specific tests for *Pseudomanas aeruginosa,* whereas there are many tests for detecting *Salmonellae* that are primarily validated for application within the food industry. Another reason for the limited uptake of specific rapid detection methods in the pharmaceutical industry is the adherence to the compendial methods that are primarily intended for reference purposes and are not prescriptive. All the compendia permit the use of other methods, provided that they are validated and give equivalent or better results. Alternatively, there may be little perceived advantage in a rapid result, because specific organisms or laboratories may simply be reluctant to change.

Alternative Approaches and Combined Technologies

Radical approaches are needed to improve the performance of pathogen detection methods, and the biggest opportunity and challenge lie in the separation and concentration of microbes during the culturing and isolation procedures.

One of the more recent techniques for this approach is immuno-magnetic separation (IMS), in which minute magnetic particles are coated with specific antibodies. The particles selectively remove the target organism from suspension and are collected simply by applying a magnet. IMS has been used for both detection and sample clean-up in both cultural and rapid detection methods. It has been shown to improve both the speed and efficiency of the isolation procedure by increasing the ratio of the target organism to the competing microflora that often give rise to false positive results (for review see Safarik et al. 1995). The IMS procedure concentrates the target organism by 2 log units. It requires samples to be incubated in a nonselective or low-selective enrichment medium for a short time (usually 18–24 hours), and an aliquot is removed for treatment.

The immuno-capture procedure is very simple and takes 15–25 minutes in a single test tube with a magnet to hold the particles. The retained particles (with the bound target organisms) are washed and resuspended *in situ* prior to testing by the final detection method of the user's choice. Commercially available kits have been certified by the AOAC International Performance Tested Program recognized by regulators.

Combining two or more rapid methods can also yield benefit in reducing the total elapsed time to obtain a result to 24 hours. For example, electrical detection methods combined with latex agglutination methods for *Salmonellae* can increase the confidence and speed of the overall detection method. The use of short (8–10 hours) pre-enrichment periods also improves the overall recovery and detection of pathogens while minimizing the interference from competing organisms (Jarvis and Easter 1989).

DNA-BASED TECHNOLOGIES

The DNA-based technologies described below are novel and future methods, and because many of them have no envisaged commercial application, the usual distinctions among pharmaceutical, food, and clinical assays are ignored. Many of the technologies are equally applicable across these categories.

Why Use Nucleic Acids to Detect Microorganisms?

In Chapter 14 Kricka considers the use of immunological techniques to detect bacteria. Nucleic acids can be used in assay formats that are very similar to those used for antibodies, but using nucleic acids gives additional advantages. The properties that make nucleic acids particularly useful in detecting microorganisms come from their unique role in biology.

The function of nucleic acids is to hold information. This makes them ideal for distinguishing between microbial species, because it is this information that defines everything about what each species is and how it differs from all other species. The presence or absence of a particular gene can be used to identify one species from many others. In some instances, the presence or absence of a specific toxin gene can distinguish between members of the same species, e.g., enterotoxigenic-producing *E. coli* identified by the presence of their toxin genes (detected using PCR [polymerase chain reaction]; Deng et al. 1996). Slight differences in the sequence of genes can be used to distinguish between different serotypes and phagetypes. However the sequence of ribosomal RNA (rRNA) cannot necessarily be used to do the same. Sequences of rRNA are particularly useful for distinguishing among species (Carlotti et al. 1997) but cannot be relied upon to separate bacteria within a species.

Another important property of nucleic acids is replication. Replication, and hence amplification, is one of the main reasons that nucleic acids are used for detection. There are two different types of amplification used in assays, one with a constant rate of increase in signal, the other with an exponential rate.

Constant rate amplification (e.g., Chiron's branched chain DNA [Lewis 1997]) boosts assay performance. However, because this boost is limited (less than 45-fold; Lewis 1998), a choice has to be made between having a more sensitive assay and a faster assay, because the increase in signal is not sufficient to have both. The trade-off made between the two is dependent on the application of the assay. Exponential rate amplification (e.g., PCR) gives a much greater boost to performance and so can increase both speed and sensitivity. However, this fast rate means that the results of exponential rate assays are very difficult to quantify and are normally limited to "presence or absence" assays, whereas constant amplification can be used in applications that require precision.

There are many enzymes that can modify, manipulate, or process nucleic acids. Some of these have already been used in assays (e.g., polymerases [PCR], ligases [LCR], restriction enzymes [RFLP—restriction fragment length polymorphism], and reverse transcriptases [TMA—transcription-medicated amplification], but many remain unused. Not only are there many more to chose from, but also their properties can be tuned by careful selection of the source organism.

A final but major benefit of detecting organisms by their nucleic acids is that nucleic acids are ubiquitous and fundamental to life. This fundamental role has ensured that research on nucleic acids has remained at the core of molecular biology. With the advent of automated DNA sequencing, more sequence data are now available from a wider and wider range of organisms. This makes the assay developers' task significantly easier. In addition, DNA is now cheap to synthesize chemically, and there exists a vast amount of data on its chemical properties, behavior in in vitro systems, and ways of modifying that behavior.

How Are Nucleic Acids Used in Assays and What Are the New Technologies?

There are two main stages in nucleic acid-based assays: (1) recognition of the target and (2) detection of the assay 's signal or output. Each step can be amplified to improve detection. Therefore, there can be amplification of the target sequence, to increase the number of target molecules to detect, and amplification of the assay signal, to increase the output from each target molecule. The new technologies are described in the context of how they fit into these two stages.

Recognition of the target by the probe

This is the first and most fundamental part of nucleic acid assays. The target sequence must be known, but the sequence of the surrounding regions is unimportant. The binding stage involves the pairing of two strands, which then hydrogen bond to each other. This binding can be DNA-DNA, RNA-RNA, or DNA-RNA. The majority of nucleic acid assays rely on DNA probes; however, there are some important exceptions where alternative forms of DNA have been developed. These exceptions include the following.

Locked nucleic acid (LNA). LNA is a DNA analogue in which changes in the chemical bonds of the furanose ring within each base decrease its normal freedom of movement and produce a more rigid form of DNA (Exiqon 1999). The furanose ring contains a methyl linker that directly joins the 2nd and 4th carbon atom in the 4 carbon sugar moiety. LNA probes show higher affinity and specificity when binding to DNA than DNA-DNA or DNA-RNA duplexes. Probes containing a proportion of LNA bases can be used as substrates for DNA enzymes such as kinases and polymerases. LNA can potentially be used to make assays more specific for their target organisms (and thus have fewer false positives) because of the higher fidelity of pairing of LNA to DNA or RNA.

Peptide nucleic acid (PNA). PNA is a completely synthetic analogue of DNA. It does not occur naturally and consists of the same bases as DNA, but linked with a peptide-like backbone instead of the conventional sugar-phosphate backbone. It was first described by Nielsen et al. (1991). The PNA peptide-like backbone is neutral, unlike the negatively charged sugarphosphate backbone of DNA. This allows PNA to bind to DNA at very low salt concentrations, unlike DNA-DNA pair bonding, which requires a high salt concentration to overcome the charge repulsion of its charged backbone. The advantage of this is that salt concentration can be adjusted so that the target DNA's double helix falls apart, allowing the PNA probe to bind instead. Producing conditions that separate the target DNA's double helix but still allow the DNA probe to bind is a major problem in developing DNA assays. It is possible that the use of PNA could allow new regions of DNA sequence to be targeted.

PNA shows faster binding, higher affinity and higher specificity of binding to DNA than DNA to DNA. This characteristic could potentially enable assays with better discrimination between genuine target organisms and closely related potential false positives. However, there is much controversy about whether PNA represents a significant breakthrough in nucleic acid technology or is an interesting idea with only a few niche applications. Because PNA is totally synthetic, no enzymes interact with it. This is an advantage in some respects, because PNA will not be degraded, but severely limits potential applications. PNA has several uses in molecular biology but, as yet, very few applications for diagnostic assays. DAKO Ltd is currently marketing PNA for use in fluorescence hybridization assays, whereby tissue sections are incubated with fluorescently labeled PNA probes and examined under a microscope.

Amplification of the target sequence

As described above, the target sequence can be amplified to increase sensitivity. PCR is the most well known example of target amplification. PCR has been used in molecular biology laboratories for many years as a tool for cloning, and its remarkable sensitivity makes it an excellent tool for detection. Reviews of some of the many applications for PCR have been published (e.g., Bryant 1997). PCR has been used by several companies to detect bacteria: Roche was the first to win FDA approval for a PCR assay (Danheiser 1994) and to produce the automated Cobas® Amplicor for detection of bacteria in clinical samples. In addition, Qualicon has devel oped the PCR-based BAX™ bacterial pathogen detector system for use in food (Qualicon Inc. 1997). Because of these established products, PCR is not considered one of the novel nucleic acid technologies. However, there are many ways of using PCR and several alternative ways of amplifying the target sequence. Examples include the following.

Restriction fragment length polymorphism PCR (RFLP-PCR). The products of the PCR amplification reaction can be analyzed by the way they are cleaved with restriction enzymes rather than simply detecting their presence or absence (Kempf 1997). Previously, only pure cultures could be analyzed using RFLP, but by specifically amplifying a small region of the cell's genome and then treating with restriction enzymes (which cut DNA at certain defined sequences), a banding pattern is produced on gel electrophoresis which can identify the type of cell (or bacteria) present. By using PCR, whole tissue extracts can be analyzed without the need for painstaking subculturing. This technique can be applied to any nucleic acid sequence.

Cleavage fragment length polymorphism. This technique is similar to RFLP-PCR, but instead of cutting the double-stranded amplification products at specific sites, the products are made single stranded and allowed to fold up into secondary structures that contain loops and hairpin bends (Lewis 1998). The folded DNA strands are then treated with cleavage enzymes that cut the DNA when the single-stranded DNA meets the hairpin bends. The resultant DNA fragments are then analyzed using gel electrophoresis, which displays the results as a pattern of bands. Changes in the sequence of the DNA are likely to change the type and size of hairpin structures produced and so change the banding pattern seen on the final gel. This

technique has been used to distinguish between clinically important strains of *Salmonella, Staphylococcus,* and others; thus, it could be transferred directly to pharmaceutical applications (Lewis 1998).

Nested PCR. In nested PCR, two separate PCR reactions are performed using two sets of primers. In the first reaction, a region of DNA is amplified as normal, by using the first set of primers. The second set of primers, which bind just inside the region of DNA that the first set have already amplified, is added, and the reaction is run again. This two-stage process is used to increase the specificity of the reaction. However, nested PCR has also been used to give several different results from one sample (Saruta et al. 1997). In this work, the first set of primers was used to amplify general prokaryotic ribosomal genes, and the second amplification used several different primers that amplified (and therefore identified) 4 different *Staphylococcus* species. This PCR application can rapidly generate a large amount of information about specific pathogen groups and their epidemiology within a single sample.

PCR on a microchip. Mosaic Technologies has used pairs of DNA primers bound to semiconductor chips to perform multiple arrays of PCR reactions that test a single sample for a variety of target sequences (Glaser 1997, Bryant 1997). Simultaneous amplification and detection of positives is possible by attaching a fiber-optic device to the chip. This system would give a rapid result in the form of a checkerboard of positives and negatives that could be used to identify and classify a range of organisms in a single reaction.

Reverse transcriptase PCR (RTPCR). This technique uses reverse transcriptase to make a DNA strand complementary to the original single-strand RNA target so that the resultant hybrid can be used as a template in the subsequent PCR reaction. This process is necessary when messenger RNA (mRNA) or rRNA (rather than the DNA gene from which it was transcribed) is being targeted. RTPCR is difficult to carry out and gives inconsistent results but can be very sensitive (Lewis 1997). Roche uses RTPCR to detect the RNA genome of hepatitis C and HPV (human papilloma virus) (Lewis 1997). RTPCR of mRNA has been used to detect *Campylobacter* (Bolton et al. 1997) because it will pick up only viable cells, whereas conventional DNA PCR would also detect dead cells.

Ligase chain reaction (LCR). LCR is similar to PCR but uses four primers and a ligase that links the two pairs to form a larger product, whereas PCR synthesizes new strands of DNA by using two primers and a polymerase. A positive result in PCR is the appearance of a new double-stranded product of a defined length; a positive in LCR results when the four primers have been completely ligated to leave two new strands, each double the length of the original primers. LCR is currently used by Abbott to detect *Chlamydia trachomatis* (Hu et al. 1996) in their automated LCx Analyzer. LCR can show greater specificity than PCR.

Primed in situ amplification (PRINS). PRINS and cycle PRINS are ways of applying amplification to samples fixed on microscope slides (Jacobs et al. 1997). Both PRINS and cycle PRINS rely on a single primer and give constant rate amplification of the target sequence, unlike PCR, which uses two primers and produces exponential amplification. The main advantage of PRINS is that the amount of fluorescence detected is directly proportional to the number of copies of the target sequence on the microscope slide. PRINS amplification is carried out at a constant temperature, whereas cycle PRINS cycles between different temperatures in a way similar to PCR (Jacobs 1997).

Rolling circle amplification. Rolling circle amplification is very similar to the way that phage DNA and plasmids are replicated in vivo (Dielgelman and Kool 1998). A target single strand of DNA is selected and converted into a closed loop by ligating its ends. Then a DNA copying enzyme (DNA polymerase) and DNA nucleotide bases are added, and the enzyme winds around the target, continuously copying its sequence to produce an unending length of DNA that has the target sequence repeated in it many times (Lewis 1998). This amplification technique is currently envisaged as being used to produce probes, but it could be adapted to amplify rRNA or mRNA sequences for analysis.

Strand displacement amplification (SDA). SDA is an amplification technique developed by Becton Dickinson that uses a DNA polymerase and an unusual restriction enzyme (Walker and Linn 1996). Conventional restriction enzymes bind to double-stranded DNA and cleave it in two; the restriction enzyme used for SDA cuts only one of the two strands in the duplex.

There are two stages in SDA, target generation and target amplification. During the target generation, DNA probes (with restriction sites) bind to a target sequence and are extended by the DNA polymerase to make two full-length DNA strands, which incorporate the restriction enzyme sites present in the original primers. Once the target strands have been generated, an amplification primer binds to each strand and is extended by the polymerase to make a copy of it that includes the restriction site. When the complete molecule is formed with a doublestranded restriction site at both ends, the restriction enzyme cuts one of the two strands at each end. The polymerase recognizes the cut site and synthesizes a new strand, simultaneously displacing the cut strand. Once the polymerase has created a new strand, it is nicked by the restriction enzyme again, and the process continues. The strands that are displaced by the polymerase can bind the primers and be used by the polymerase to produce more strands. This process continues to amplify more and more target sequences.

Becton Dickinson has developed a high-temperature protocol that can give results within 30 minutes. Further developments include an RNA-compatible version, a method to give quantitative results (Walker et al. 1996), and a protocol for in situ

applications suitable for immunohistochemistry. Current applications include a diagnostic test for *Mycobacterium tuberculosis* with a sensitivity of fewer than 10 genomes within 20 minutes (Walker and Linn 1996).

Transcription-mediated amplification (TMA). TMA uses two enzymes (RNA polymerase and reverse transcriptase) and two primers, one of which contains the promoter sequence for the RNA polymerase. The promoter-primer binds to single-stranded rRNA and is used by the reverse transcriptase to produce a complementary DNA strand (see Figure 9.1). The RNA strand is then degraded by the RNase H activity of the reverse transcriptase (it only degrades RNA when RNA is hybridized to DNA). The second primer binds to the DNA copy and is extended by the reverse transcriptase to produce a double-stranded DNA molecule. The RNA polymerase recognizes the promoter in the first strand of the two DNA strands to be produced and synthesizes hundreds of RNA copies. Each of these RNA copies can then bind the second primer, which is used by the reverse transcriptase to produce a DNA full-length copy. The reverse transcriptase then degrades the RNA strand of the hybridized RNA-DNA pair, leaving single-stranded DNA. This DNA strand (which has the same sequence as the original rRNA molecule) binds the promoter-primer, and a new DNA strand is synthesized, leaving a double-stranded DNA molecule that contains the RNase polymerase promoter sequence. This triggers another round of replication whereby the RNA polymerase again makes hundreds of RNA copies. Within an hour, TMA rapidly produces many billions of amplicons.

The amplicons are detected using Gen-Probe's hybrid protection assay (HPA). HPA relies on a selection reagent and DNA probes labeled with acridinium esters. The probes are added to the sample and allowed to bind to the target. The selection reagent is then added, and it inactivates the acridinium ester of any probes that have not bound to their target but does not affect any probes that have found a target sequence. When the activation reagent is added, light is emitted only if the target is present.

TMA has been developed by Gen-Probe to detect *Mycobacterium tuberculosis* in clinical samples (Gen-Probe 1999). Assays for HIV (human immunodeficiency virus) and *Chlamydia trachomatis* have also been produced. TMA has advantages over PCR: it is isothermic and so does not require the complex temperature cycling necessary for PCR; everything happens in one tube; and because TMA uses labile rRNA, the material is less likely to become contaminated. However, TMA is suitable only for the detection of RNA.

TMA is directly applicable for determining presence or absence of pathogens in the pharmaceutical industry.

Nucleic acid sequence-based amplification (NASBA). NASBA is an isothermic RNA amplification technique developed by Organon Teknika.

NASBA has several stages. The first stage is sample preparation; RNA and DNA present in the sample are bound to silicon dioxide particles, washed, and then eluted from the particles to leave purified nucleic acid.

The amplification stage uses reverse transcriptase, RNase H and T7 RNA polymerase. A primer binds to the single-stranded RNA template and is extended by the reverse transcriptase to make a DNA copy. The RNA strand of the duplex is then degraded by the RNase H, leaving a single DNA strand. The second primer (which contains a recognition site for the T7 RNA polymerase) binds to this strand and is extended by the reverse transcriptase to produce a double-stranded DNA product. This double-stranded product now contains the recognition site for the polymerase and so is used by the polymerase to produce many RNA strands. Each of these can bind the second probe and make a complementary DNA strand; the RNA is then degraded by the RNase H, and the DNA strand is bound by the first probe and used to make another DNA strand, resulting in a second double-stranded DNA molecule. Because this molecule contains the T7 RNA polymerase recognition site, more RNA copies are made, and the cycle starts again. The amplified products are detected either in a qualitative form by gel electrophoresis or in a quantitative form by electrochemiluminescence (Romano et al. 1997).

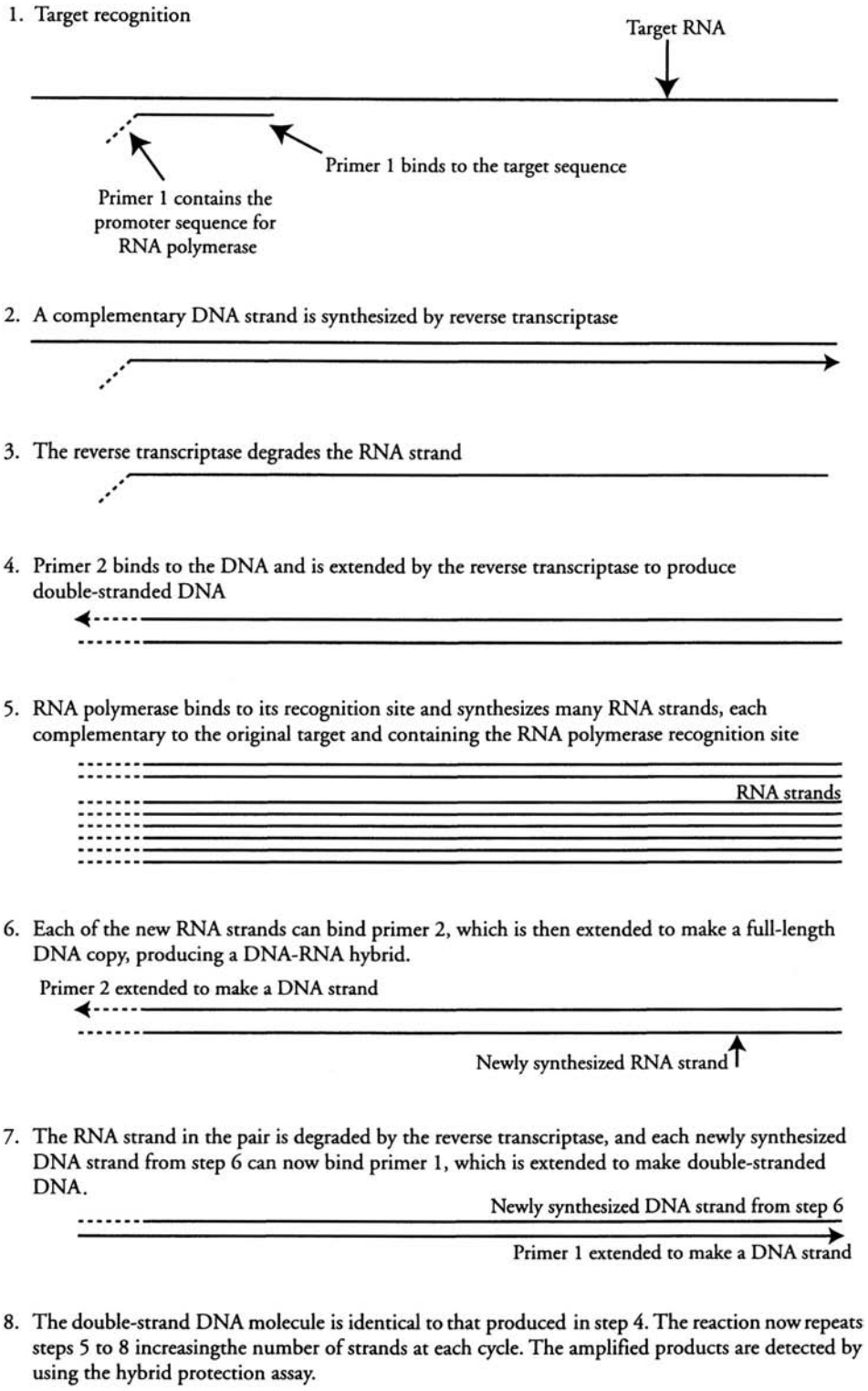
NASBA has been used to detect HIV (Gobbers et al. 1997), *Mycobacterium leprae* (van der Vliet et al. 1996), and type *Mycoplasma pneumoniae* (Walker and Linn 1996). NASBA is very similar to TMA, the main difference being that NASBA uses three enzymes, whereas TMA uses two.

Detection of the assay signal

Nucleic acids have no conventional enzymic activity and so cannot be viewed directly. Therefore, a translation step is required to produce an output. The results of nucleic acid assays are most commonly visualized by using small intermediate molecules (haptens) that are attached to the probe during its synthesis. Either these haptens are fluorescent dyes that can be viewed directly or they act as receptors and allow the binding of antibody-conjugated or streptavidin-conjugated enzymes. The bound enzymes are then detected by means of colorimetric or chemiluminescent substrate systems. This is very similar to the way antibody-based assays are visualized. However, new approaches to detection are being developed.

Hybrid capture. A novel way of using antibodies with nucleic acid probes has been developed by Digene Inc. In its hybrid capture assay, DNA probes bind to their RNA targets, and the resultant hybrid is detected using antibodies that recognize the double helix of the RNA/DNA hybrid (Teutonico 1998). Tubes are coated with these antibodies, which capture the hybrids from solution. The hybrids are then detected by using more of the same type of anti-hybrid anti-body, this time conjugated to an enzyme rather than bound to the tube. The enzyme that ends up bound to the tube wall is detected by using a

Figure 9.1 Transcription-mediated amplification.



chemiluminescent substrate. Digene uses this system to produce assays for HPV, but in theory the assay could be used for any bacterial target.

Chemiluminescent DNA probes. A more convenient approach than detecting DNA probes with enzyme-labeled antibodies is to directly couple the DNA probe to an enzyme label. This removes the need for a separate "translation" stage

and allows faster assays. Life Technologies conjugates alkaline phosphatase (an enzyme commonly used as a label in antibody assays) directly to DNA probes for human chromosomes and markets them for forensic and paternity applications (Glaser 1996). The alkaline phosphatase-labeled probe-target pair is then visualized by using a chemiluminescent substrate. Gen-Probe uses enzyme-labeled DNA probes to detect *Mycobacterium tuberculis* and claims that this assay is much faster and more sensitive than the conventional methods.

This approach produces simpler, faster, and more sensitive assays for the clinical market and, with the development of thermostable forms of alkaline phosphatase, is highly likely to become common in nucleic acid-based applications.

Simultaneous amplification and detection for PCR. The results of PCR reactions are usually displayed as bands on a gel after gel electrophoresis. This is a laborious and time-consuming process. New techniques are available to speed up the detection of PCR products by monitoring their production during the amplification reaction (Lewis 1997).

Roche has developed an advanced thermal cycler that uses capillary tubes for reaction vessels (Rasmussen et al. 1998). The products of the reaction are detected by using a fluorescent dye (SYBR Green 1 [Rasmussen et al. 1998]) in conjunction with a fluorimeter. SYBR Green 1 fluorescence is greatly enhanced when it binds to double-stranded DNA, so as more DNA is produced by the amplification reaction, more SYBR Green 1 binds, and so the amount of fluorescence detected increases. Use of this system makes it possible to perform PCR and determine the results within 20 minutes.

Roche also uses fluorescence resonance energy transfer (FRET) to detect and monitor the synthesis of specific sequences within their PCR reactions. In FRET, an additional pair of fluorophorelabeled probes is added to the reaction mixture. These probes are designed to bind immediately adjacent to one another on one of the amplified target DNA strands. When the probes are illuminated with light of a single wavelength, only probe A's fluor F1 is excited; this then emits light at a different wavelength which is absorbed by probe B 's fluor F2 and re-emitted at a third new wavelength. The results are determined by the amount of light emitted at probe B's specific emission wavelength.

The efficiency of the transfer of light from probe A to probe B is critically dependent on the distance between them. This efficiency falls at a rate proportional to $1/r^6$ where r is the distance between the probes. If the specific target is absent, the PCR reaction does not produce the necessary DNA strands and so the two probes are separated within the solution, thus giving a very low output at F2's wavelength. If the target is present, they bind adjacent to each other and so the output at F2's wavelength dramatically increases. Because the fluorescence of the reaction can be continuously monitored, real-time detection of specific sequences within the reaction is possible.

A similar process to FRET, homogeneous time-resolved fluorescence (HTRF), patented by Packard Company in 1998 (described in Park et al. 1999), produces a homogeneous assay detection system that could be applied to the rapid detection of microbial nucleic acids.

An alternative system that does not rely on sophisticated proprietary equipment is called Sunrise, developed by Oncor (now taken over by Intergen) (Lewis 1997). This system uses PCR primers that have hairpin loops with a fluorophore and a quencher conjugated to each loop on each primer. If no target is present, the primers remain looped, and no light is detected because the quencher absorbs all emitted light. If the primers are incorporated into PCR products, the loops are opened, allowing the fluorophore to emit when it is illuminated. Once the PCR reactions are completed, the tubes can be quickly read in a fluorimeter.

Atomic force microscopy. As described by Kricka in Chapter 14, the atomic force microscope (AFM) can be used to detect the binding of antibodies to their respective antigens. The AFM has also been used to detect the binding of one DNA strand to another (G.U. Lee et al. 1996). The "target" strand is bound to a fixed surface and the "probe" strand is attached to the sensor of the AFM. When the strands are not touching, there is no force. As the strands are moved together, a force is measured as the two DNA molecules compress together. The two surfaces are then slowly separated. First, an intrachain attractive force is detected as the strands begin to uncoil. This is followed by an interchain attractive force as the Watson-Crick base pairing is stretched and broken. For a set length of DNA probe, the interchain force is proportional to the number of bases that pair and so indicates the similarity between the known and the test sequence. Although the technology is extremely complex, it does allow real-time monitoring of a very small number of molecules (Aleen and Thornton 1997).

Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) DNA detection. This process uses mass spectrometry to determine DNA sequence. A mass spectrometer vaporizes DNA and accelerates it through a vacuum by using an electric field (Alper 1998). The time it takes to reach a detector is measured and used to calculate its size and hence its sequence. The technique is sensitive enough to detect differences in the mass of DNA strands of as little as 0.03%. MALDI-TOF can analyze DNA samples in less than 2 seconds; this speed allows rapid throughput but requires extensive purification of the DNA from other macro-molecules. Its most likely application is in rapid sequencing.

Gel electrophoresis. This technique identifies DNA or RNA by the way it moves during electrophoresis. Nucleic acids are negatively charged by virtue of their sugar-phosphate backbone and so will slowly move toward the positive electrode when placed in an electric field. Their movement is slowed by a 3-dimensional molecular lattice formed by an agarose gel. The process allows nucleic acids to be distinguished by their size (the smaller the molecule, the farther it moves) and their shape (the more tightly folded the molecule, the farther it moves). Therefore, specific sequences can be identified by how far they

move relative to a set of internal standards. This technique is very commonly used in molecular biology labs and is often used to identify the products of PCR reactions (D.H. Lee et al. 1996). Transgenomic Inc. (Inside Industry 1998) is developing novel chromatographic matrixes so that gel electrophoresis can be used for heredity studies, forensics, and paternity suits.

FISH on human chromosomes. Fluorescence in situ hybridization (FISH) is a long-standing technique of visualizing DNA probes bound to tissue sections or other slide-mounted samples under the microscope. The DNA probes are conjugated to a fluorescent dye and are viewed under ultraviolet light. New advances include the use of 5 fluorophores, both singly and in combination, allowing 25 different targets to be identified and distinguished from each other simultaneously (Lewis 1996). This technique allows the identification of all 24 human chromosomes in the same microscope field of view. FISH has not yet been accepted as a clinical tool by the FDA, but it could be used for a rapid count of specific organisms or groups of organisms.

Amplification of the assay signal

Most nucleic acid amplification techniques amplify the target and not the signal. However, techniques for signal amplification have been developed, and many of the principles involved are suitable for both immunological and nucleic acid formats. Some examples are described below.

Branched chain DNA. Chiron's branched chain DNA (Lewis 1997) is a technique whereby a single DNA probe binds to its target and then binds a many-branched DNA oligonucleotide complex, which in turn binds many reporter probes. These probes are conjugated to alkaline phosphatase, which is visualized by using a chemiluminescent substrate. The branched chain amplifier consists of 15 DNA spokes, each with 3 binding sites (Lewis 1998). In the absence of "amplifier," one probe binds one enzyme; with the branched intermediate complex, one probe can bind up to 45 enzymes. As described above, this type of amplification does not give a vast increase in sensitivity, but the output is proportional to the number of probes bound and so can produce quantitative data.

Dymontor Dendrimers. This technique is similar to branched chain DNA, but instead of using a single large branched intermediate between the enzyme and probe, it uses many monomeric DNA molecules that bind together to build up a very large polymeric structure. Each monomeric form consists of double-stranded DNA with two single strands at each end which can bind to other identical monomers. The more monomers that bind, the more single strands are available at the complex's edges for binding, and so the rate of binding increases over time. The resultant branched DNA structure can be visualized by using a final treatment of monomers labeled with conventional binding ligands (such as biotin) or with enzymes such as alkaline phosphatase. The technology is proprietary to Polyprobe (Lewis 1998).

Q beta replicase amplification. This technique relies on two separate RNA probes that bind adjacent to one another on an RNA target sequence. Each probe contains half of the recognition site of the RNA replicase enzyme from the bacteriophage Q beta and a sequence complementary to the target sequence (Tyagi et al. 1996).

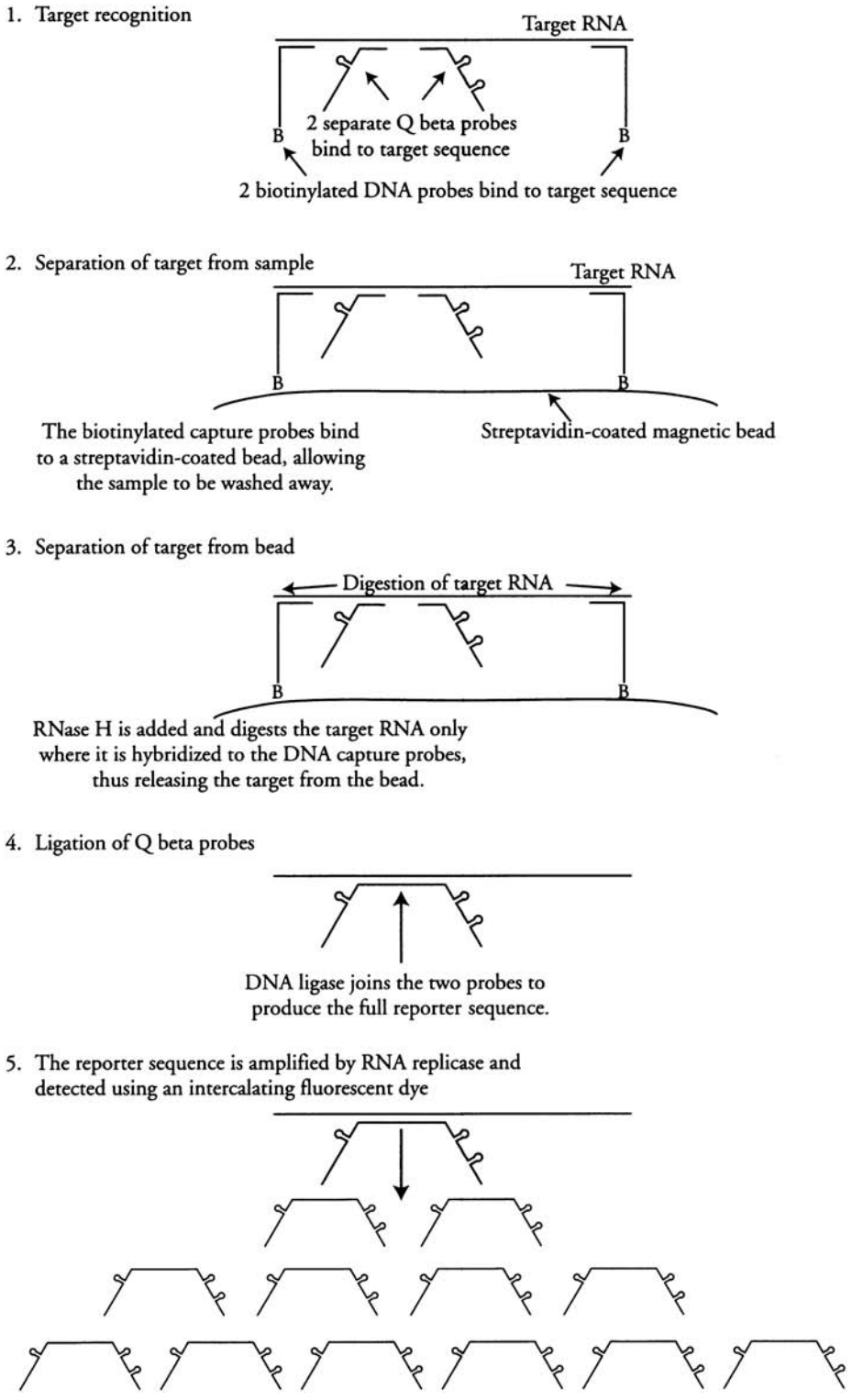
The RNA Q beta probes are added to the sample along with a pair of biotinylated DNA "capture" probes (see Figure 9.2). All four probes bind to the target RNA sequence. The two capture probes then bind to a streptavidin-coated magnetic bead, via the biotin-streptavidin linkage, which allows the sample matrix to be washed away, leaving the target sequence with all four probes still attached. Ribonuclease H is then added; it digests the target RNA, but only where it has hybridized the two DNA capture probes. This releases the target RNA, the two RNA probes still being bound into the fluid phase. The magnetic bead is then extracted, and a DNA ligase is added which joins the two RNA probes. This joining event is vital because the RNA replicase enzyme that is used to amplify the RNA probes can work only with the fully intact recognition site. RNA replicase is added and then produces many billions of copies of the ligated probe sequence in an isothermic reaction lasting 30 minutes. The presence of the amplified probe sequence indicates the presence of the initial target sequence (Tyagi et al. 1996). The sensitivity of this assay is reported to be 40 target RNA molecules; in the Tyagi et al. study, the amplified sequence was detected using autoradiography (Tyagi et al. 1996). A more convenient technique is to use an intercalating fluorescent dye, which allows simultaneous amplification and detection (Burg et al. 1996).

Q beta replicase amplification has three main differences from PCR: the probes are RNA; the probe molecules are amplified, rather than the target sequence; and Q beta replicase has a "capture" stage on a solid phase that allows purification of the target sequence from the potentially inhibitory sample matrix.

Q beta replicase has been used by Gene-Trak to detect *Chlamydia trachomatis* (Stefano et al. 1997), *Mycobacterium tuberculosis* (Smith et al. 1997), and *Legionella pneumophila* (Stone et al. 1996).

Liquid crystals. Liquid crystals have been used to detect the binding of antigens to anti-bodies. The binding events cause the crystals to change the intensity of light that they transmit. This process does not require any electroanalytical equipment. The researchers have not attempted to link DNA to the liquid crystal films; if this were possible, it would be a very convenient way of displaying a positive signal. Work on this technology is likely to produce some interesting applications (Gupta et al. 1998).

Figure 9.2 Q beta replicase amplification.



Biosensors. This topic is covered in Chapter 14. The potential for DNA probes bound to chips or other electronic devices is extremely exciting and could lead to some completely new formats, applications, or instruments for nucleic acid assays (Knight 1998, Wrotnowski 1998).

WHY AREN'T MORE NUCLEIC ACID ASSAYS BEING USED?

Many nucleic acid technologies are extremely clever, sophisticated, and well designed. However, not all make it to the marketplace, and relatively few are successful. Why is this so? Of the many possible reasons, one of the most important is the level of sensitivity required. If an assay of presence or absence is considered, then the assay cannot miss a single bacterial cell within a set sample volume. Very often, the bacterial cell may be damaged by the sample itself, so that its RNA levels are decreased, and it may possibly have damage to its genomic DNA. It is very difficult for any assay to detect with 100% sensitivity and specificity that single damaged cell within a (for example) 200 g sample. A resuscitation period in growth media is normally required to allow the bacterial cell to repair any damage and to allow it to replicate to reach high enough numbers (e.g., 100–1,000 cfu/ml) to have a realistic chance of unfailing detection. When a resuscitation period is included, the advantage of the nucleic acid assay over the conventional cultural assay becomes less obvious; for some, it is no longer worth the time and effort required to change to the new method.

Apart from sensitivity, the sample itself can be a problem. Nucleic acid assays can have the most exquisite sensitivity and specificity when performed on pure cultures, but they can fail completely when bacteria are present in a large volume of a complex matrix. Frequently, the most complex problem is to isolate the target bacteria, which are usually present at extremely low concentrations. Filtration can be used for very large volumes (e.g., 20 l of rinse water), but this detracts from the ideal of an assay that is extremely rapid and simple to perform. Various ways of separating bacteria or DNA from the sample matrix have been developed: e.g., using soluble polymers to aggregate water-borne bacterial cells (Bennet et al. 1997) and magnetic beads coated with DNA to capture nucleic acid within the sample (De Palma 1996, 1997). Despite these attempts, bacterial detection within some sample matrixes still remains a formidable problem.

A more fundamental problem arises when there is a conflict in the classification of bacteria between the original cultural methods and the new nucleic acid technologies. This is slowly changing, because of new epidemiological studies based on bacterial genotype rather than phenotype (Stackebrandt and Goodfellow 1994).

The prospect of having to validate a new method can increase the unwillingness of the pharmaceutical industry to change to new methods. If there is an overwhelming advantage in changing to a new method, it will happen, but the less apparent the benefit, the less likely the change.

If the technologies described in this chapter appear inapplicable to the pharmaceutical industry, it must be remembered that PCR initially seemed unlikely to have any application outside the research laboratory because of its many perceived problems. These problems included: the expense and size of the equipment needed to run the assay; the skill level required to perform the technique; the specificity of the results; the time-consuming technique used to visualize the results; the problems of contamination and the inhibitory effects of the sample matrix. However, thanks to a lot of development time and money, the majority of these problems have now been solved in the following ways. Simple-to-use handheld PCR machines the size of a paperback book have been developed—e.g., Cepheid's MicroBE™ (Dutton 1998). The MicroBE can extract the DNA, perform the heating and cooling cycles, and detect the final amplified products. It uses disposable cartridges that contain all the reagents required. This type of equipment could bring nucleic acid assays to the often quoted but seldom realized "doctor's office" environment. The skill level needed to perform PCR has been reduced as more and more semi-automated systems have been developed; Roche's Cobas™ and LightCycler are examples. PCR specificity has been increased in many ways—for example, by improving the DNA polymerase (the enzyme that amplifies DNA in PCR reactions). Roche has developed a "hot-start" version of Taq polymerase (Avramovic 1996). Previous versions of Taq polymerase began to amplify DNA before the temperature had risen high enough to ensure full specificity. The new "hot-start" version, as its name suggests, will only begin to synthesize DNA when the temperature is high enough to guarantee specificity. The visualization technique has been greatly simplified from the original gel electrophoresis, so now some systems can monitor the concentration of amplified products during the amplification reaction, and other systems detect the amplified product in a simple one-step procedure afterward. The perils of contamination have been lessened by use of a novel artificial form of thymidine, one of the four bases that make up all DNA sequences (Avramovic 1996). This new form is incorporated into all the amplified products made by the PCR reactions. When a new PCR reaction is set up, the enzyme AmpErase™ is added, which destroys any DNA molecules that contain the artificial thymidine (and hence any contaminating DNA from previous reactions) but ignores all normal, natural DNA. The AmpErase enzyme is then itself destroyed by the high temperature of the PCR reaction. Although PCR is still inhibited by many different sample matrixes, advances have been made. Qualicon has developed new sample preparation protocols which decrease sample inhibition and allow PCR-based detection of salmonella in foods (Qualicon Inc. 1997). Sample inhibition is the main problem yet to be solved.

CONCLUSION

It is almost inevitable that nucleic acid assays will replace classical microbiology for the detection and identification of microorganisms. The reason for this is the quantity and quality of information available from nucleic acids. As described in the

first part of this chapter, the biological role of nucleic acids is to store the information that defines what each microorganism is and what differentiates it from all other organisms. Detecting and identifying a microorganism by its nucleic acid produces much more information than does measuring the binding of an antibody to its antigens or determining what different nutrients it requires to multiply. Nucleic acids give information on the organisms genotype instead of its phenotype. Consequently, the information is definitive, not descriptive, and will stop uncertain identification. The benefits are in the speed and confidence of detection and identification. These factors will be the push that makes nucleic acid assays standard in the industry.

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10
Alternative Technologies for Sterility Testing

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The use of compendial standards for the regulation of the identity, purity, potency, quality, packaging, labeling, and storage of parenteral drugs is familiar. Among the attributes that contribute to the quality of a sterile product is, obviously, documented evidence of its sterility. To this end, the sterility testing procedure is highly regulated. Sterility testing was first documented in the British Pharmacopoeia in 1932. The United States Pharmacopeia (USP) added sterility testing in 1936, and the methods described then remain largely unchanged (d'Arbeloff 1988).

The original sterility testing procedure, although relatively simple, was clearly based on sound scientific ideals and captured all of the necessary components found in today's protocol. The Tests for the Sterility of Liquids protocol was simpler than what we practice today in that it only called for incubation of the product in aerobic broth and it only described a direct transfer method for liquids. Yet, despite its relative simplicity, this early compendium included instructions for verification of media sterility; steps to take in the event that the material being tested rendered the media turbid; quantities of material to be tested, based on the container and batch size; a dilution scheme for material containing preservatives; and retest criteria in the event that a sample was found to be positive for growth (U.S. Pharmacopeia 1932). Since 1936, employment of standardized test cultures, addition of anaerobic incubation conditions, the membrane filtration technique, and the idea of testing devices purporting to be sterile, as well as liquids, were added to the chapter. Yet the basic concepts remained the same.

False positive sterility test results are a major concern to pharmaceutical manufacturers. Further, a reduction in the number of false positive results can, by some estimates, equate to cost savings to a pharmaceutical company of tens or hundreds of thousands of dollars per year if one takes into account the cost to investigate and retest the product or to destroy product that may be sterile (Reisman 1998). Given the implications of a positive sterility test result, extreme consideration was given to the elimination of false positive results stemming from personnel or environmental influences. In the past 20 years, two changes to the traditional sterility testing procedure provided valuable alternatives for those dependent on sterility tests. The first of these changes was the development of the closed membrane filtration system introduced in 1974 by Millipore Corporation (for details, see the Web site http://www.millipore.com/analytical/technote/ micre/brochures/eu379/ eu3791.html). Millipore's Steritest™ device addressed the market's desire to reduce the number of false positive results due to open product transfer and the membrane manipulation involved in conventional membrane filtration methods. The design of the system allows the filtration, media addition, and incubation steps of the sterility test to be self-contained. The only step left exposed for human or environmental contamination is the sampling step, when the product is extracted from its container. Regardless, closed membrane filtration has dropped the sterility test failure rate substantially (d'Arbeloff 1988). The second change to the traditional sterility testing procedure was the introduction of the isolator chamber. This system provides a microenvironment that allows aseptic manipulation without human intervention. Isolator systems protect the article being tested from contamination due to faulty aseptic technique by the technician or a poorly controlled environment (Anonymous 1997). Isolator systems range in size from a glove box to an entire room, depending on the size and needs of the testing laboratory. Isolator systems have been shown to be effective, and more widespread use may be hampered only by their cost and complexity (d'Arbeloff 1988). Market-driven regulatory agency acceptance of both of these methods resulted in their inclusion in the compendium.

Today, there are alternative technologies that may be used for sterility testing. Some of these are new; others have been available for some time but have been used in industries other than pharmaceutical sterility testing. Like Steritest and the isolator systems, successful incorporation of these products into pharmaceutical testing laboratories by using creative and

scientifically sound validation protocols will drive their acceptance by regulatory agencies. Six such alternate technologies are described herein.

ALTERNATE TECHNOLOGIES: CLINICAL ORIGINS

The first three of the alternate technologies described here have similar histories. Starting in about 1971, a manufacturer of clinical diagnostics developed an instrument to automatically examine blood culture bottles for evidence of microbial growth. Most of the market accepted this new method of detecting septicemia. Other companies followed with automated blood culture instruments of their own. Automation, convenient and labor saving, began to replace the manual method of inspecting each individual bottle for turbidity or hemolysis. Some companies creatively approached the problem of detection of microorganisms in a fluid system by inventing new technologies and engineering novel instrumentation to accomplish the task. What these technologies have in common is that each is capable of reporting the presence or absence of microorganisms that can grow in the media provided by the manufacturer and used in their specific instruments.

The proven success of these technologies in the detection of microorganisms in blood cultures led to the natural extension from clinical to industrial applications. Candidates for the industrial samples included any product in which testing for the presence or absence of microorganisms was required and a suitable quantity of the product could be transferred into the bottles. Examples include cosmetic and personal care products, foods, and pharmaceutical articles.

Each of these three systems offers faster time to detection than conventional manual methods, automated record-keeping capabilities, continuous monitoring, labor savings, and elimi nation of the transfer step required by the USP for turbid samples. Adaptation of these instruments to specific industrial samples and performance varies for each of them. Individuals interested in exploring these instruments for industrial sterility testing should be cautioned that there is a licensing issue that governs the specific applications allowed for each instrument. Be sure to discuss your application with the manufacturers prior to making an investment.

BACTEC® System

BD (Becton, Dickinson and Company, United States) was the first company to automate the labor-intensive blood culture test in clinical laboratories. The company's instruments have evolved from invasive, batch-style radiometric detection of CO_2 gas produced by microorganisms (1971) to a continuous-monitoring system that uses a fluorescent CO_2 detection system (1992). In all cases, the technology relies on CO_2 production to detect positive samples. The newest technology marketed by BD, the BACTEC, a fluorescent detection system, is based on the presence of a pH-sensitive fluorescent-CO_2 sensor poured into the bottom of each bottle. Samples are recognized as positive for growth detection on the basis of computer algorithms that identify an increasing rate of change as well as sustained increase in CO_2 production (Nolte et al. 1993). The different sizes of the BACTEC 9000 series, which uses the fluorescent technology described, range from 50 to 240 septum bottles.

This technology has potential for use in a variety of industrial applications. The BACTEC has been described as useful in testing food products such as tomato juice, carbonated beverages, and ultra-pasteurized milk, and for pharmaceutical raw materials, detecting low viable counts of aerobic and anaerobic bacteria and fungi (Hammann and Kunert 1989). The BACTEC has also been used for blood sterility testing by incorporating the instrument into a protocol that mimicked the USP compendial method for sterility testing of pharmaceutical articles. In that study, the blood components were tested in response to a reported rise in the number of bacterially contaminated units in blood banks that could result in transfusion-transmitted sepsis, and the BACTEC was shown to be a useful tool (Alvarez et al. 1995). At this time, growth media formulated specifically for industrial applications is not available for use on the BACTEC instrument.

The documented history of the BACTEC's ability to detect a wide variety of microorganisms in clinical samples and the outlook presented by researchers in the pharmaceutical and food industries make it a prime candidate for use in industrial microbiology laboratories, including pharmaceutical sterility testing.

ESP® Detection System

The ESP system (TREK Diagnostic Systems, United States) uses a technology based on the detection of gas consumption and/or production by microorganisms as a function of growth. The instrument continuously monitors changes in the headspace pressure of a sample bottle by utilizing pressure-sensitive transducers. As changes in the headspace pressure occur because of organism growth, a microprocessor performs calculations of these changes. If one of several algorithm parameters is met, a positive growth response is indicated by the system. The design of the instrument accommodates both septa and widemouthed bottles. Instrument capacity ranges from 128 to 384 bottles.

To date, the ESP system has been tested in several industrial applications. The first is as an alternative to the traditional sterility testing method described in Sterility Tests in USP 23 (U.S. Pharmacopeia 1995). Media bottles containing modified aerobic

and anaerobic broth were used. Modifications of the formulae did not affect the growth of the microorganisms. Rather, components were added as a supplement to encourage all organisms to either produce or consume gas, so that they are detected by the system. In addition to more usual pharmaceutical products and devices, the ESP system has also been proven for testing spent tissue culture media and components as a means of evaluating the purity of the cultured cells and actively metabolizing chondrocytes used for autologous transplant (Meszaros et al. 1996b; Buxton et al. 1996). A second application was quality assurance testing of cosmetic and personal care products. In this application, widemouthed bottles containing media capable of neutralizing preservative systems used in this industry and a solid growth platform for microorganisms were developed. The media is a modified trypic soy broth with 0.07% lecithin and 1.5% Tween® 80. In this case, the manual methods being replaced are described by Cosmetic, Toiletry and Fragrance Association (CTFA), the FDA, Bacteriological Analytical Manual (BAM), AOAC International and the USP. These methods have been the standard in the cosmetic and personal care product industry for many years (Lenczewski et al. 1997). The third application developed involves testing the commercial sterility of ultrahigh-temperature processed canned food products (Meszaros et al. 1996a). In each of these industrial applications, the ESP system performed as well as or better than the conventional methods to which it was compared. Currently, the ESP system is marketed in the clinical and veterinary markets.

BacT/Alert®

The BacT/Alert (Organon Teknika, United States) also originated in the clinical blood culture market. Like the BACTEC, this instrument 's operation is based on the production of CO_2 by active metabolization of microorganisms. Each bottle contains a colorimetric CO_2 sensor, at the bottom, covered with a membrane that is permeable only to carbon dioxide. As the metabolizing microorganisms generate carbon dioxide, the CO_2 passes through the membrane. A sensor pad saturated with a pH-sensitive solution changes from green to yellow as the level of CO_2 in the bottle increases. The detection system is noninvasive; the bottles are loaded into the instrument and monitored by a small LED (light-emitting diode), which shines on the pad and emits a light signal in the presence of CO_2 (Alpert 1990). The BacT/Alert is available with either a 120 or 240 sample capacity.

The BacT/Alert has made headway in several industrial markets. In the food industry, the instrument has been tested for determining commercial sterility of foods such as aseptically processed tomato paste and pudding, and low-alcohol cocktail beverages; results have been positive (Robison 1994; Anonymous 1996). The BacT/Alert has also been evaluated for use in culturing donor blood and determining the sterility of samples from musculoskeletal and cardiovascular tissue (Weiss and Wilson 1997). It was found to be sensitive enough to detect low levels of several species commonly isolated from donor samples. Weiss describes a 7-day sterility method using BacT/Alert that proved as reliable as 14-day sterility results using the USP method. The study validated the use of BacT/Alert as an alternative to traditional sterility testing methods.

As with the previously described automated instruments, there is potential application of the BacT/Alert in the future in any market where there is a need for sterility testing. Of the three instruments with a clinical origin, BacT/Alert has had the broadest impact on the industrial sterility testing markets. Organon Teknika received 510(k) approval for use of BacT/Alert for platelet screening on February 15, 2002.

ALTERNATE TECHNOLOGIES: NONCLINICAL ORIGINS

The remaining three alternate technologies for sterility testing do not have clinical origins. One was originally developed for the food industry and later adapted for pharmaceutical testing. The others were developed with a variety of applications in mind.

Bactometer®

Electrical impedance variations as a result of the metabolic activity of growing microorganisms were studied as early as the late 1890s (Eden and Eden 1984). Since that time, impedance microbiology has become widely accepted as a technology well suited for rapid microbiological methods (for further details see Chapter 7). The Bactometer (bioMérieux, United States) is a rapid system that was developed for use in the detection and enumeration of microorganisms in foods and cosmetics. It has more recently been evaluated as an alternate sterility testing method. The Bactometer is an incubator-type instrument within which disposable modules are placed. Each module consists of 16 test wells. Each Bactometer processing unit holds eight modules, for a total of 128 samples. The principle of operation is based on the measurement of an electrical parameter of the growth medium, impedance. Impedance is defined as the resistance to the flow of an alternating electrical current through the medium. When modules containing growth medium are inoculated, microorganism growth causes the ionic constituency of the medium to change. This change is measured through two electrodes at the bottom of the wells. The system is monitored by a computer equipped with an algorithm that calculates the detection time in the presence of cells in the sample. The time

required for the microorganism in the sample to reach the instrument's limit of detection correlates with the original bacterial number present, as determined by the SPC (standard plate count) method (Dalmaso 1998).

The Bactometer is widely used in the testing of a variety of food products and raw materials. It has also been evaluated for sterility testing of sterile products by the membrane filtration method. Antibiotic solutions were filtered through membranes that were subsequently transferred into the wells of the Bactometer modules for incubation. The Bactometer was found to be a reliable method for routine sterility testing (Dalmaso 1998).

ScanRDI®

The ScanRDI (Chemunex, United States) (also described in Chapter 4) is new to the market-place. This system is based on direct fluorescent labeling of viable microorganisms, coupled with an ultrasensitive laser counting system. There are three steps to the procedure. First, the samples are put through membrane filters, which retain any microorganisms present. Second, the viable microorganisms are labeled directly on the filter by using a nonfluorescent fluorescein derivative that is taken up by the cells and cleaved by esterase activity within the cell to give the fluorescent product, fluorescein. Finally, the membrane is transferred to the analyzer, which scans by laser the entire surface of the membrane and detects individual viable microorganisms by their fluorescent signal (Wallner et al. 1997). This instrument is the closest to providing real-time results for process control. The faster analysis time enables manufacturers to identify potential contaminants in raw materials and process intermediates earlier, reducing the number of failed batches and their associated cost. This device is unique in that it eliminates the time-consuming cell cultivation step, which is required in all of the technologies previously described. Elimination of this step not only saves time, but also allows detection of stressed microorganisms and slow growers based on its direct measurement at the cellular level.

The ScanRDI is currently being used for real-time microbial enumeration in pharmaceutical water systems and is reported to be at least as sensitive as the standard plate count method (Wallner et al. 1997; Brailsford et al. 1998). It has been evaluated for use in pharmaceutical applications including sterility testing, biological indicators, bioburden testing, and fermentation control.

The Electronic Nose

Instruments that detect and digitally characterize smells by electronic means, using an array of chemically sensitive sensors, are known colloquially as electronic noses (Gardner and Bartlett 1999). The digital response to a complex odor may be used to identify or describe that odor in a qualitative way and has many potential applications, including the simultaneous detection and identification of contaminants. For example, the BH 114 instrument (Bloodhound Sensors Ltd., United Kingdom), has 14 sensors that respond in real time to give a unique pattern of responses to the order of different bacteria. The different parameters, such as peak height, rate of adsorption, rate of desorption, and area under the curve, are processed using statistical methods or neural network techniques to give a "digital fingerprint," thus identifying the bacteria producing the odor. A 4–6 hour incubation period is claimed to be sufficient to produce a characteristic odor, and organisms such as *E. coli, Pseudomonas aeruginosa, and Staphyloccocus aureus* can be distinguished with 95% confidence match. The technique can diagnose *Helicobacter pylori* infections and can identify tuberculosis noninvasively. This new technology has yet to be validated for pharmaceutical applications. Future miniaturization of the technology can produce simpler portable devices. Modifications to produce new techniques, such as pulse spectroscopy, can give responses similar to GC-MS (gas chromatography-mass spectrometry) but in only a few seconds (Gibson et al. 2000). It is conceivable that such technologies could be applied to sterility test methods to detect and identify contaminants simultaneously; however, the technology is in its infancy.

REGULATORY ACCEPTANCE AND THE FUTURE FOR STERILITY TESTING

As the number of these novel methods for sterility testing increases, the industry is faced with the issue of how to validate and implement the new methods for routine use in microbiology laboratories. Regulatory acceptance is difficult to predict, but significant progress on harmonization, validation, and adoption of alternative microbiological methods is being made (see Chapter 13, on regulatory issues, and Chapter 12, on validation). Although the compendial methods are quite specific for sterility testing procedures, they do allow for equivalent methods to be used, as long as those methods can be shown to provide equivalent or better results (U.S. Pharmacopeia 1995; European Pharmacopoeia, 1998). It is at this point that the definition of "equivalent" becomes a matter of interpretation.

The existing sterility test is a qualitative test based on the ability of surviving organisms to recover and grow. Haberer and Mittelmann demonstrated in Chapter 3 the highly variable and fastidious nature of microorganisms; the sterility test can never give 100% assurance of the absence of viable organisms, yet is has been considered the best standard for testing requirements.

Harmonization efforts try to resolve the differences in test methods of the three major global pharmacopeias (mainly issues of procedures, media formulations, and incubation times and temperatures), but they are missing the point. It is time for a fresh start, to look at the problems and challenges that face the industry in ensuring consumer safety. Akers (2001) has reasoned eloquently for a pragmatic approach to the assurance of sterile products. He suggests that the major advances made over the past 20 years in processing technology have outstripped the testing methodologies that have now outlived their usefulness. The bioburden resulting from aseptic processing or sterilization is, by definition, zero, but the test methods applied are designed to show growth where there should be none. At its best the sterility test detects only high-level gross contamination. Frustrated microbiologists around the world inist that "there must be a better way," yet industry and regulators are reluctant to challenge the inadequacies of the test. A greater reliance and application of in-process controls under parametric release procedures would give a better assurance of safety; these ideas will surely become more widely considered and accepted.

A system based on the rational assessment of hazards and risks should provide the correct framework and motivation for change based on well-reasoned argument and fact. Gillet (1996) described many established systems and procedures for safety assurance. These are currently focused on chemical, environmental, and personal hazards and their control. Little attention has been directed at qualifying the real microbiological hazards and risks in industrial manufacturing and product supply. A careful but realistic review of those real microbiological hazards is desperately needed.

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11
Limulus Endotoxin Test

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DISCOVERY

Howell (1885) observed that the blood of *Limulus polyphemus,* the horseshoe crab, formed a solid clot when withdrawn from the crab. Loeb (1902) noted that when the blood cells of *Limulus* were collected and exposed to foreign substances, they coagulated. These were the first papers detailing various aspects of coagulation with reference to amebocytes, which are the only circulating cells found in the blood of *Limulus.*

In 1964 Levin and Bang (1964a, 1964b) reported that *Escherichia coli* endotoxins and a marine *Vibrio* induced the extracellular coagulation of *Limulus* blood. Although endotoxin did not cause coagulation of the cell free serum, clotting activity could be restored by the addition of amebocytes to the serum. The following conclusions were drawn from this study: amebocytes were required for coagulation, disruption of the amebocytes enhanced the reaction, and quantities of endotoxin were possibly inactivated by the coagulation process. Amebocytes coagulating in the presence of bacterial endotoxin is not limited solely to *Limulus* but has been demonstrated in the oyster, crab, and lobster also. In a subsequent study, Levin et al. (1970) developed a sensitive assay for endotoxin using the lysed amebocyte from *Limulus polyphemus.* As little as 0.0005 μg of endotoxin per milliliter could be detected, and the rate of the reaction depended on the concentration of endotoxin. Yin et al. (1972) refined Levin's original endotoxin assay to detect picogram amounts of endotoxin and demonstrated that the lipid A part of the lipopolysaccharide was responsible for lysate gelation.

Comparison of Blood Coagulation in *Limulus* and Mammals

A most exciting aspect of the increasing knowledge of the basis for the reaction between amebocyte lysate and endotoxin has been recognition of the remarkable similarities between the biochemistry of coagulation in *Limulus,* a primitive and ancient invertebrate, and mammalian blood coagulation. Most of the knowledge in reference to the biochemical aspects of coagulation in horseshoe crabs has been provided by a series of outstanding Japanese researchers, who have studied all four of the species of the horseshoe crab (Morita et al. 1984). All of the enzymes that have been identified are serine proteases, and at least three serine proteases are required for the activation of *Limulus* blood coagulation by endotoxin (Nakamura and Levin 1982). It has been reported that the same enzymatic inhibitors block mammalian and horseshoe crab coagulation (Young et al. 1972). In fact, some of the synthetic substances designated for the assay of human coagulation factors also are excellent substrates for the components of the coagulation system in *Limulus* (Torano et al. 1984).

Preparation of *Limulus* Amebocyte Lysate

The horseshoe crabs are either captured by commercial fisherman (in nets) or by persons on the beach or in shallow water during the crab mating season. The crabs are then either placed in a tank of ocean water for a few hours, to release excess dirt and sand, or are scrubbed with a brush in tap water. When the crab has air dried, it is flexed and placed in bleeding racks. The muscular hinge between the cephalothorax and abdominal region is then scrubbed with iodine or 70% alcohol, and a sterile, endotoxin-free 18-gauge needle is inserted into the heart chamber. Hemolymph can then flow freely into an appropriate container (usually a 250 mL glass centrifuge bottle), which is either empty or contains about 70 mL of an anticlotting agent, such as 0.125% N-ethylmalemide (NEM) in 3% sodium chloride solution (Levin and Bang 1968). The NEM is a sulfhydryl

inhibitor that stabilizes the amebocyte cell membrane sufficiently to permit washing. The hemolymph that is mixed with NEM can be centrifuged when time allows; however, the hemolymph that is not mixed with NEM must be centrifuged within 15 or 30 minutes or the hemolymph will clot in the bottle. The large white cells are gently sedimented in the 250 mL tubes by centrifugation at 300 G for six minutes. After discarding the blue (hemocyanincontaining) supernatant, the amebocytes are washed with warm 3% sodium chloride solution. The cells are then transferred to 50 mL graduated centrifuge tubes and washed with warm sodium chloride solution. The amebocytes are then lysed by adding sterile water for injection, to cells in a 2:1 ratio and vortexing vigorously at least three times daily for one or two days. Finally, the amebocyte lysate is separated from the cellular debris by centrifugation at 1200 G for ten minutes, the supernatant is removed, and the lysate is stored at 4°C. Most lysate preparations remain stable for at least nine months at 4°C. The lysate is then tested for potency by using EC-6 endotoxin and freeze-dried. The expiration date of freeze-dried lysate is about five years.

EARLY REGULATORY INTEREST IN AN ALTERNATIVE TO THE RABBIT PYROGEN TEST

In early 1970 the personnel in the Division of Biologic Standards (DBS), National Institutes of Health (NIH), were made aware that a new test was being developed for detecting endotoxin. This test used the lysed amebocytes from the *Limulus polyphemus* (horseshoe crab) and could detect nanogram quantities of endotoxin (lipopolysaccharide, LPS) located in the cell wall of gram-negative bacteria. Laboratory personnel were performing the rabbit pyrogen test on at least four licensed biological products per day and decided to incorporate the Limulus Amebocyte Lysate (LAL) test when testing certain biological blood products. These products were 5 and 25% Normal Serum Albumin (Human, NSA), Purified Protein Factor (PPF), Immune Serum Globulin (ISG), and Antihemophilic Factor (AHF) (Hochstein et al. 1979).

The first several batches of lysate were prepared at the Johns Hopkins University. After about five months of trial and error, it was determined that the *in vitro* test really was of value in detecting endotoxin. Because the DBS laboratory was about 150 miles from the ocean, horseshoe crabs were difficult to obtain, so it was decided to have a crew spend one week each year, for the next several years, at the NASA base in Chincoteague, Va., bleeding horseshoe crabs. About 800 to 1,000 crabs were bled per week, and the crew would return to the laboratory with several liters of lysate and 200 to 300 live crabs. These crabs were put into three-tier fiberglass lobster tanks (Bergson Products,Warren, R.I.). Each tank was 10 feet long and 20 inches deep. The tanks from bottom to top were 6, 5, and 4 feet wide. This arrangement provided total visibility of the interior of the bottom two tanks and also provided steps for reaching the top tank. Each tank contained 7 inches (depth) of synthetic seawater (Instant Ocean). A pump connected to the bottom tank pumped the salt water to the top tank. From there it ran out the overflow into the middle tank and then back to the bottom tank. Each tank held between 70 and 100 crabs, depending on their size. If the tanks were full and there were extra crabs, they were put into plastic boxes and placed in the refrigerator at 4°C to 8°C. If the crabs were kept moist, they would live in the refrigerator for at least six months without food or water. The crabs did not move in this environment as long as the light was off, but when the light in the refrigerator went on, the crabs responded to this stimulus by moving their tails. The crab tanks were housed in the rabbit holding room. Following a pyrogen test, the rabbits were sacrificed and their livers removed. These livers were rinsed in tap water, sliced, and fed to the crabs. The crabs thrived on this diet.

Each crab in the tanks was numbered, so that a bleeding record could be kept. The crabs were bled semiannually . If they were bled more frequently, the crabs produced hemolymph but very few amebocytes, indicating that it took at least six months for the horseshoe crabs (in captivity) to regenerate their normal amebocyte count.

In 1972 the LAL test began to be widely used in the medical community. At the same time, the U.S. Food and Drug Administration (FDA) received complaints that some people were using lysate to diagnose septicemia, meningitis, and urinary tract infections. The FDA decided that regulations had to be written to control the sale and use of this product. Because LAL was a blood product and the expertise for using it was in the Division of Control Activities, it was decided to assign this product to the newly created Bureau of Biologics (BoB); that replaced the DBS.

FDA's Experience with Bacterial Endotoxin

It soon became apparent that in order to determine the lysate sensitivity, a standard endotoxin was needed. In 1974 the FDA contracted with the University of Montana to prepare 30 g of purified endotoxin from *E. coli* O1 13:H10:K negative (Rudbach et al. 1976). This bulk endotoxin was labeled EC and was stored in a desiccating jar at room temperature.

In 1976 a portion of this bulk EC endotoxin was used to prepare lot EC-1. Before freeze-drying, NSA (human) at 0.1% concentration was added as a stabilizer. This small lot, consisting of several hundred vials, lasted about one year. It was then decided to prepare a large lot of endotoxin in the same way EC-1 was prepared. This lot, EC-2, consisted of 1,500 vials, each containing 1.0 µg of EC endotoxin. After extensive data were collected on this very stable standard preparation, it was decided to assign a unit value to the preparation. Most control authorities agree that it is better to use units of activity rather than a concentration based on dry weight in expressing the strength of standard preparations. On the basis of a collaborative

study involving the FDA and several licensed LAL manufacturers, a value of 5.0 endotoxin units (an endotoxin unit is a measure of potency) per nanogram (EU/ng) was assigned to lot EC-2 (Rastogi et al. 1979). The only criticism of lot EC-2 was that it contained NSA (human) that might bind endotoxin. To eliminate this hypothetical problem, in 1980 lot EC-3 was prepared as a pilot lot with no fillers or stabilizers. After several months of use, this lot appeared satisfactory, and lot EC-4 was prepared in the same way. After EC-4 was freeze-dried (with no filler or stabilizer), not only did the vials appear empty, but it was quite difficult to dissolve all the endotoxin into the water used for reconstitution. This lot was depleted in 1981.

Each new lot of endotoxin created problems in that the in-house standards had to be revalidated to reflect the new endotoxin; therefore, it was decided that to avoid the necessity of recalibration each year or two, a large lot of endotoxin would be prepared that would last 10–15 years.

This large lot of EC-5 endotoxin was prepared from the EC bulk powder. Because lot EC-2 was the best that had been prepared, it was decided to prepare lot EC-5 in the same way. A licensed lysate manufacturer was contracted to freeze-dry 30,000 vials from a single bulk. The protocol used for lot EC-5 was prepared jointly by the U.S. Pharmacopoeia (USP) and the FDA; lactose and polyethylene glycol were specified as stabilizers (Hochstein et al. 1983).

This lot was stored at −20°C at the USP facility in Rockville, Md., and was distributed by the agency as Endotoxin Standard Lot F. Several hundred vials of this material were stored at the Center for Biologics Evaluation and Research (CBER) facility (Kensington, Md.) and distributed upon request to licensed manufacturers as the U.S. Standard Endotoxin lot EC-5. There has been a great interest in endotoxin testing by using both the amebocyte lysate test and the rabbit pyrogen test. An endotoxin rabbit study (Hochstein et al. 1983) indicated that it required about 10 EU/kg of EC-5 to elicit a 1°C rise in a rabbit. A similar study in humans to determine the threshold to pyrogenic stimulation by EC-5 was the next step. This study was done at the Clinical Research Center in New Orleans, La. (Hochstein et al. 1994). Human male volunteers were divided randomly into 5 groups of 12. Each group was given one intravenous injection of lot EC-5 at a level of 0, 2, 4, 8, or 16 EU/kg of body weight. Oral temperatures were taken and recorded every 15 minutes for 8 hours. The pyrogenic variations of the U.S. Standard Endotoxin in humans over the test period were determined. The results indicated that there was a direct correlation between endotoxin units per kilogram administered and temperature rise. The threshold pyrogenic dose (1.0°F rise in 50% of volunteers) in this study was approximately 4.1 EU/kg. This was the first report that describes the human dose response to the intravenous administration of a pyrogen-free water control and four dose levels of U.S. Standard Endotoxin, Lot EC-5. Backed by this information, the FDA guideline on use of the lysate test states that the maximum human dose of endotoxin should be 5 EU/kg. This dose may cause a slight fever in about half of the patients, but it should not cause shock or death. EC-5 was the best endotoxin lot; however, by 1995 it was rapidly being depleted, so a replacement was needed quickly. The USP stated that if CBER furnished the bulk EC powder and some expertise on the preparation of EC-6, the USP would have it prepared at the National Institutes of Biological Standards and Controls (NIBSC). In late 1996 about 60,000 vials, each containing 10,000 EUs, were prepared. This lot was labeled EC-6 (Poole et al. 1997). After an extensive collaborative study involving more than 20 testing facilities worldwide, this lot was accepted as the World Health Organization (WHO) standard endotoxin and is being distributed by the USP as lot G endotoxin. This lot should last until about 2007.

Licensing the LAL Test

The FDA is responsible for licensing all *Limulus* or *Tachypleus* amebocyte lysate manufacturers. To be licensed, a manufacturer must complete the FDA forms for an establishment license for the manufacture of biological products. The licensing procedure comprises the following. After all the forms and samples are received, an ad hoc committee is established to review the submission in detail. While the paperwork is being reviewed, the product samples are distributed to specific laboratories for moisture, sensitivity, and sterility testing. When the paper review and sample testing are complete, an on-site inspection of the manufacturing facility is scheduled at a time when the appropriate personnel will be present for answering questions. One of the inspectors is usually a committee member with expertise in the testing of the product. The inspection involves not only checking the physical plant, but also reviewing daily records, including standard operating procedures for each production step. The validation records of equipment such as freeze-dryers, flow hoods, and autoclaves are also reviewed. If any exceptions to the federal regulations are noted during the inspection, the inspector discusses them with management before leaving the facility. The manufacturer then has the responsibility to reply in writing to each observation. The licensing committee reviews all data and submits a recommendation to the CBER director regarding licensure.

A manufacturer that has been licensed must submit to CBER each lot of lysate prepared for sale. If the lot passes, the manufacturer is notified that the lot is released and may be marketed. The FDA has recently specified that the lot to lot release approval can be waived if some parameters (e.g., track record) are consistent. However, if a later problem develops (e.g., sterility, potency) the lot to lot release approval can be reinstated. In 1976 three lysate manufacturers applied for a license to produce and market LAL. They completed all necessary requests for an establishment license for the manufacture of biological products and were licensed in 1977. Since then twelve additional U.S. companies and one Japanese company have been licensed.

LAL Licensed Manufacturers

Today there are only five licensed amebocyte lysate manufacturers remaining, all in the United States. These are Associates of Cape Cod, Inc., Baxter Healthcare, Charles River Endosafe, Haemachem, and BioWhittaker. Due to an ever increasing regulatory burden, low market growth (increased competition), and difficulty securing horseshoe crabs, one or two of these may soon relinquish their licenses.

After a manufacturer is licensed, a sample of each lysate lot must be submitted to CBER for release and must be accompanied by two forms. One is a protocol that lists all the manufacturer's test results, including sterility and chemical and physical data. The second form is the raw data potency sheet that shows the test results on the contents of four final containers in parallel with four replicates of the FDA reference lysate. Both lysates are tested with U.S. Standard Endotoxin Lot EC-6. Because the sensitivity of the reference lysate and the potency of Lot EC-6 are known, one can determine if the testing laboratory is in control. Both the reference lysate and endotoxin are supplied to LAL manufacturers for their in-house release testing. FDA laboratories test the sensitivity of each lot of lysate before it is released. If the test value is within twofold of the manufacturer's sensitivity test, the lot is released. If the testing is outside the twofold range, four more samples are tested; if the test value is still outside the range, the manufacturer is notified of the problem and requested to retest more samples. If the manufacturer is unable to match FDA results, the lot is rejected, and the manufacturer is notified that the lot cannot be released. The testing of samples and the review of the product protocol usually takes two to three weeks.

Each manufacturing facility (both manufacturing area and horseshoe crab bleeding areas) is inspected at least every two years. During these inspections, the inspectors review many standard operating procedures (SOPs) and current good manufacturing practices (cGMPs); interview persons directly involved with day-to-day activities; review books containing data of laboratory testing of the product; review all temperature charts for deviation from set ranges; make sure that all released products are segregated from unreleased products; review records indicating that the crabs were returned to their natural habitat within 48 hours after bleeding; check to see if all processing steps have been dated and initiated as completed; and determining if any new major personnel, equipment, or renovations have taken place since the last inspection and if these were reported to the FDA as major changes. If any exceptions are noted during the inspection, an annual inspection is performed until these exceptions are corrected.

COMMERCIALIZATION

In addition to the interest by the DBS in the LAL test, numerous research laboratories, both academic and commercial, in the United States and Japan initiated research projects involving LAL (and its Asian counterpart, *Tachypleus* amebocyte lysate, or TAL). Significant among these were Travenol Laboratories; Mallinckrodt, Inc.; and the Woods Hole Oceanographic Institution in the United States, and Seikagaku Corporation (and their university collaborators) in Japan. Out of this group came four licensed LAL manufacturers (Travenol Laboratories, licensed manufacturer for internal use only; Mallinckrodt, Inc., no longer in the LAL business; Associates of Cape Cod, Inc., spun off from the Woods Hole Oceanographic Institution; and Seikagaku Corporation, who merged with Associates of Cape Cod, Inc., in 1997). Along with Fredrik Bang, Jack Levin, several U.S. university laboratories, and those involved with the FDA, researchers associated with these groups are a Who 's Who of LAL.

Of the private organizations interested early in LAL, four groups figured prominently with the FDA in the commercialization of this product. These were Associates of Cape Cod, Inc.; Mallinckrodt, Inc.; Microbiological Associates; and Travenol Laboratories. Associates of Cape Cod, Inc., founded by Stanley Watson specifically to manufacture and sell LAL, was the first company licensed by the FDA. Mallinckrodt and Microbiological Associates soon followed. Stanley Watson, Marlys Weary, and Phil Griffiths of Mallinckrodt, along with one of us (Hochstein) and Ed Seligmann at the FDA figured prominently in formulating a consensus set of rules for manufacturing and quality control for LAL. James Cooper, who developed methods to apply LAL to the testing of pharmaceutical products while a researcher with Hochstein at the FDA, later went on to found Endosafe, Inc. Although the LAL reagent became commercially available in 1977, research on the components, reactivity, and application of LAL continued in earnest. Jack Levin at the Marine Biological Laboratories continued his investigations into clinical applications of LAL (Levin et al. 1970). Stanley Watson and his group including one of us (Novitsky) at the Woods Hole Oceanographic Institution worked on environmental applications of the LAL test (Watson et al. 1977), while Watson's group at Associates of Cape Cod, Inc., worked on improving sensitivity, stability, and specificity (Sullivan and Watson 1974). Weary at Baxter Travenol was quite active in pharmaceutical applications and regulations (Weary and Baker 1977). Over the years other companies decided to commercialize LAL; 13 were granted establishment licenses for LAL by the FDA. These included, in addition to those already mentioned, then Microbiological Associates, a division of Dynasciences Corporation; CooperBiomedical, Inc.; Difco Laboratories; Millipore Corporation; Diagnostic Isotopes, Inc.; Worthington Diagnostic Systems, Inc.; Marine Biologicals, Inc.; Neumann Biotechnologies, Inc., and Endosafe, currently a division of Charles River Laboratories. In Japan, Seikagaku Corporation commercialized a TAL reagent

in 1978 but did not decide to pursue a U.S. license until later, receiving the first foreign license to manufacture LAL (actually TAL) in 1995. From the beginning LAL has been a fascinating product, as witnessed by the number of companies getting into the business. The reality, however, is that the world market is relatively small, which is one reason more than half of the companies receiving FDA licenses for LAL are no longer in the business.

Evolution of the Test Methodology

The first LAL test methodology approved by the FDA was the gel-clot assay. This assay is based on the observations of the clotting phenomenon of horseshoe crab blood by Levin and Bang (1964a). In their description of the assay, Levin and Bang refer to the LAL reagent as *pre-gel,* an extract of horseshoe crab amebocytes. This assay was later modified for commercialization through the addition of salts, for activation, and stabilizers, so the reagent could be freeze-dried. The assay was also standardized with respect to time and temperature and later to reactivity with a standard endotoxin preparation. Otherwise, the gel-clot assay is little changed from its original description. Today this version of the LAL test is still the most widely used, because of its simplicity. The gel-clot assay, however, is subjective owing to the manual reading of the test, and its semiquantitative nature. Because of this, much research has been conducted on alternative test methods (Novitsky 1993). Of the dozens that have been described since 1970, only two beside the gel-clot assay are in widespread use today: the kinetic turbidimetric method, first described by Levin and Bang (1968) and commercialized by Associates of Cape Cod, Inc., and the chromogenic method, first described by Nakamura et al. (1977) and commercialized by Seikagaku Corporation. In the United States, however, BioWhittaker, Inc. (then M.A. Bioproducts), was the first to introduce a chromogenic LAL approved by the FDA. Other methods worth noting include the colorimetric test and the end-point turbidimetric assay. For a short time, Travenol Laboratories used the colorimetric assay (Nandan and Brown 1977). This assay employed a protein determination of the insoluble gel-clot formed during the LAL reaction with endotoxin. The colorimetric assay eliminated the subjectivity of the gel-clot test and made the assay quantitative. It was, however, somewhat cumbersome and was replaced (at Travenol) by a chromogenic assay. The first commercially available quantitative assay was the end-point turbidimetric assay introduced by Worthington Biochemical (Bondar et al. 1979). This test employed an ordinary spectrophotometer with a 360 nm filter and read the turbidity that developed after a fixed period of incubation at 37°C. The need for precise timing and the lack of inexpensive multi-sample incubating spectrophotometers made this assay unpopular. Although the first chromogenic assays also required precise timing, unlike the turbidimetric assay, the end-point chromogenic test could be stopped with the addition of acid and the result read later. Addition of acid to a gel-clot or turbidimetric assay lead to the dissolution of the clot or turbidity. The first kinetic assay was a turbidimetric LAL test described by Levin and Bang (1968). This assay was most likely not readily commercialized because of lack of adequate equipment for performing kinetic assays on multiple samples. With the advent of machines like the MS-2 (Abbott Laboratories) adapted for use with the LAL assay as described by Jorgenson and Alexander (1981), incubating microplate readers by Albaugh and Chandler (1982), and the purpose-designed LAL-4000 by Novitsky et al. (1987), kinetic LAL assays became feasible. They were commercialized with the introduction of an LAL specifically designed for kinetic turbidimetric testing by Associates of Cape Cod, Inc. The final assay to be developed was the kinetic chromogenic test. This assay development was difficult for several reasons. Early chromogenic substrates were invariably contaminated with endotoxin. To circumvent this problem, the end-point assay consisted of two steps. The first involved incubation of a sample (containing endotoxin) with LAL under optimal conditions for activation. The second step involved adding the chromogenic substrate and allowing color to develop. In this two-step assay, the first step, activation, was slow (about 10 to 15 minutes) compared to the second, color development (1 to 3 minutes). Because the activation part of the assay was separated from the development part, it did not matter if the substrate was contaminated with small amounts of endotoxin. To make a single-step kinetic assay, however, the substrate needed to be as endotoxin-free as possible. Two companies involved in early research with the chromogenic assay, Kabi AB and Seikagaku Corporation, succeeded in producing clean chromogenic substrates. In addition, other factors that affected the performance of the reagent were worked out (Lindsay et al. 1989), and a kinetic chromogenic assay was finally commercialized by BioWhittaker.

Current Technology and Automation

Today the majority of LAL tests are still conducted by using the gel-clot method. However, use of one of the automated, kinetic methods is growing rapidly. The kinetic test employing a turbidimetric LAL is still in widespread use. The LAL-4000 has since been replaced with the LAL-5000, and other machines have appeared on the market, including the Toxinometer® (Wako), the ATI-6000®, the PUR-32 (Pyroquant), the Pyros® KinetiX, and various incubating microplate readers. At least two LAL manufacturers now market LAL reagents especially formulated for kinetic turbidimetric assays.

The chromogenic assay is also in widespread use, both as an end point and a kinetic test. The LAL successor to Kabi AB (Chromogenix) markets Chromo-LAL®, a kinetic LAL test manufactured by Associates of Cape Cod, Inc., but incorporating a Chromogenix (IL Laboratories) substrate. Newcomer Charles River Endosafe also has a kinetic chromogenic reagent that

employs a Chromogenix substrate. Seikagaku markets a dual-use (kinetic and endpoint) reagent, Toxicolor, with an additional diazo option for reading the end-point version. Associates of Cape Cod, Inc., also markets a similar reagent under license from Seikagaku but employing lysate from *Limulus* rather than *Tachypleus.* BioWhittaker continues as the U.S. market leader, with its chromogenic LAL coupled with a microplate reader and computer software system that greatly facilitate data collection and analysis. All manufacturers are working on new software that will be compliant with current federal regulations (Part 11).

Although users seem satisfied with the current LAL assay choices, improvements continue to be made in reagent performance (sensitivity, specificity, stability), instrumentation, and automation. BioWhittaker, for example, has teamed with Beckman to offer a complete liquid handling/assay system for those users with large testing requirements.

Seikagaku was first to offer an endotoxin-specific LAL. Because all LAL, unless specifically altered to remove or inhibit the glucan-reactive factor G, reacts to various degrees with beta 1, 3 glucans, there is a potential problem with false positive tests. Fortunately, false positive tests related to glucan contamination are relatively rare in pharmaceutical products. Problems do occur in clinical testing, dialysis, certain medical devices, and processed blood products. Currently two manufacturers offer products purported to "block" glucan activity when added to their lysates. It is likely that endotoxin-specific as well as glucan-specific lysates will be developed.

INDUSTRY ACCEPTANCE

Replacement of the Pyrogen (Rabbit) Test

Because a standardized test for pyrogens using rabbits was not implemented until 1942 with publication of USP XII, the pyrogen test was relatively new when Levin and Bang were making their initial discoveries regarding LAL in the 1960s. It wasn't until 1955 that the actual pyrogenic dose of endotoxin was determined in the laboratory of Otto Westphal (Eichenberger et al. 1955). Westphal was also able to confirm that the minimal pyrogenic dose of a purified *Salmonella* endotoxin was comparable in rabbit and humans. A dilemma for regulators, however, is that not all endotoxin species are equal as pyrogens, and, perhaps more important, not all pyrogens are endotoxins. For example, the threshold pyrogenic dose in human and rabbit was tenfold less for *Salmonella typhosa* endotoxin than for *E. coli* endotoxin, whereas *Pseudomonas* was 50 times greater (Greisman and Hornick 1969). Arguments ensued over selection and care of rabbits, assay protocol, and pyrogen standards. These have continued to a lesser degree to the present time. In 1978 the Health Industry Manufacturers Association (HIMA) established, in a collaborative study, the pyrogenicity of a well-characterized, readily available endotoxin standard from *E. coli* 055:B5 (Dabbah et al. 1980). This study established that, at the 95% confidence interval, a laboratory will attain a 50% pass or fail result at concentrations of *E. coli* 055:B5 (Difco) above 0.098 ng/mL (0.98 ng/kg dose at 10 mL/kg). This translated roughly to an LAL test pass or fail limit of 1.0 ng/mL using the same standard. HIMA concluded that if a laboratory could demonstrate an LAL test failure rate significantly greater than 50% using LAL sensitive to 0.1 ng/mL of the 055:B5 endotoxin, the test could be considered equivalent to the pyrogen (rabbit) test. A Parenteral Drug Association (PDA) *Limulus* Amebocyte Lysate Task Force also concluded that the USP pyrogen test approaches a 1 ng sensitivity and that this level could serve as a reference point for an endotoxin limit in small-volume parenterals. Large-volume parenterals were not addressed. The PDA also extended their review to the then current FDA reference endotoxin standard, EC-2, and found that the threshold pyrogenic dose (TPD) for EC-2 was about 1.0 ng/kg. This was significant because the FDA guideline recommended 0.5 ng/kg as the limit for endotoxin concentration in parenteral drugs.

The acceptance of a reliable standard as well as a reasonable endotoxin limit was considered essential for industry acceptance of the LAL test. In 1983 the FDA published the results of its collaborative study on a reference endotoxin standard. This study also established a potency for the new EC-5 standard (from *E. coli* O113:H10:K-) of 10 EU/ng. Earlier the FDA had established the EU to facilitate comparison of different endotoxin preparations. In 1985 the HIMA published the results of another collaborative study (Pearson et al. 1985). This one compared several control standard endotoxins, those used routinely by quality control and assurance laboratories, with the then current FDA reference standard EC-5. The *E. coli* O55:B5 standard was also included in this study, and all preparations were assigned EUs according to the reference standard. This study confirmed the similarity in potencies between the reference standard and O55:B5 and also added validity to the selection of 5.0 EU/kg as the limit of endotoxin (activity) in devices, parenteral drugs, and biological products.

After publication of the FDA's draft guideline for validation of LAL in 1983 and the USP's Bacterial Endotoxins Test chapter in 1985, industry acceptance was virtually complete. Implementation, however, except for in-process control, followed slowly.

Sensitivity, Reproducibility, Sample Interference

Sensitivity of the LAL test has always been greater than that of any other endotoxin detection test. From an industry standpoint, this was not an endearing feature of the assay. For those accustomed to the pyrogen test, which was not designed

to be quantitative, a positive test was a failure. With the LAL test, validation of sensitivity, use of graded standards, and dilution of sample became absolutely necessary to properly compare LAL with rabbit results. The first commercially available LAL had an average sensitivity of 0.025 ng/mL (equivalent to 0.25 EU/mL). Thus, to use this LAL as a pass-fail test, one had to dilute the drug 1:2 to meet the endotoxin limit of 5.0 EU/kg based on a dose of 10 mL/kg. As manufacturing of LAL improved, and with the introduction of the alternative methods, LAL sensitivity improved. Typical gel-clot sensitivities today are 0.03 EU/mL, and chromogenic and turbidimetric results reach 0.005 and 0.001 EU/mL, respectively.

The reproducibility of the test has always been within those expected with other enzyme assays employing biological standards and dilutions. Thus the error associated with the gel-clot test employing twofold dilutions of sample or standard is accepted at plus or minus one dilution. The error limits with the quantitative methods are usually tighter, approximating those associated with ELISA-type assays. Thus coefficients of variation (CVs) around 10% or less are readily achievable, with recovery of endotoxin added to samples approaching 25%.

Sample interference remains the single largest problem associated with the use of LAL. With the rabbit pyrogen test, sample interference was never controlled; i.e., the test did not require validation using known amounts of endotoxin added to samples. Therefore, little information on sample interference in the pyrogen test is available. With LAL, sample interference is easy to measure and is quite common (Dawson 1996, Dawson 1997a). It could be said that all samples tested with LAL when compared to pure water probably interfere with the test to some degree. Basically, any component in the sample that affects the LAL enzyme cascade or the availability of endotoxin can and will interfere with the test. Common examples are pH, ions, proteins that bind endotoxin, and detergents. Interference can manifest itself both as an inhibition of the reaction—i.e., lower than expected endotoxin concentration measured—or as enhancement—i.e., higher than expected endotoxin concentration measured. Apart from the use of buffer or other pH adjustment, dilution is the method of choice for elimination of sample interference. If dilution is used, however, sensitivity of the LAL becomes more important, especially if the endotoxin limit of the sample under test is close to the sensitivity of the LAL reagent being used. Thus the value of the very sensitive LAL available today can be seen when samples with extreme interference are encountered.

Non-Endotoxin Pyrogens

From the beginning, industry was concerned that the LAL test was not an adequate replacement for the pyrogen test, because not all pyrogens are endotoxins. Thus pyrogens such as viruses, proteins (bacterial toxins), and various chemicals would be missed by the LAL test. As it turned out, however, endotoxins are the most ubiquitous pyrogens and those most likely to be encountered in pharmaceuticals. This was shown convincingly by the FDA (Twohy et al. 1984) and is now given only slight attention during the validation phase of new drugs. Thus the rabbit pyrogen test has not gone away completely but is required at least once during the development of a new drug to prevent the occurrence of nonendotoxin pyrogens. Recently, an *in vitro* pyrogen test has been introduced that uses human monocytes or cell culture (Novitsky 2002). This test, once adequately validated, may prove to be a viable replacement for the rabbit.

False Positives

Less important than missing a nonendotoxin pyrogen or, for that matter, obtaining a false negative result, is finding a false positive. False positives affect the user by requiring repeat tests, explanations to the regulatory authority, and possibly even discard of product. With the exception of certain types of glucans, however, the LAL test is quite specific for endotoxin (Roslansky and Novitsky 1991). LAL reactive glucans, although often associated with cellulose, are (1,3)-D-glucans, which are mostly of fungal origin. They are found in cellulose because this material is readily colonized by fungi during processing. Since all glucans, whether biologically reactive or not, are basically similar chemically, LAL reactive glucans co-process with the cellulose and end up in various concentrations in the final product as a contaminant. Fortunately, the glucan reaction with LAL has been thoroughly documented and therefore can be readily addressed if it occurs. Chemical procedures and even LAL reagents that have been modified so as not to react with glucans can be used if glucan reactivity is suspected. Of course, the presence of (1,3)-D-glucan in a product that does not include this biologically active glucan as a component is indicative of a contamination event. Therefore, a positive LAL test can be viewed as a tool for controlling contamination in addition to testing for endotoxin. Several facts act in the favor of pharmaceutical manufacturers: (1) LAL positive tests are rare on products produced in GMP facilities, especially if the water used has already passed an LAL test; (2) very few LAL positive tests on final products result from glucan, with the exception of products filtered using cellulosic filter media and certain cellulose-containing medical devices—e.g., certain artificial kidneys; and (3) it is very easy to differentiate between glucan and endotoxin if a positive LAL test occurs. Because of these facts, it would be wise for a pharmaceutical manufacturer to use an LAL that *is* reactive for glucans when in-process testing and when evaluating raw materials for suitability. In certain validated cases, i.e., for the release of artificial kidneys that have been shown to shed LAL reactive glucans that may not affect patient safety—a nonglucan reactive LAL is warranted. Users should be worried, however, that certain LAL formulations containing

Zwittergent®, although showing reduced reactivity to glucans, may also be poorly reactive with lipid A and environmental endotoxins low in polysaccharide content (Roslansky and Novitsky 1991). It is also too soon to know whether the "glucan blockers" sold by some lysate manufacturers to remove glucan reactivity affect the LAL's ability to detect naturally occurring endotoxin.

FDA GUIDELINES

In 1973 (FDA 1973), the FDA announced that *Limulus* amebocyte lysate (LAL), derived from circulating blood cells (amebocytes) of the horseshoe crab *(Limulus polyphemus),* is a biological product (U.S. Food and Drug Administration 1973). As such, it is subject to licensing requirements as provided in section 351 of the Public Health Service Act (42 U.S.C. 262). Since 1973, LAL has proved to be a sensitive indicator of the presence of bacterial endotoxins (pyrogens). Because of this demonstrated sensitivity, LAL can be of value in preventing the administration or use of products that may produce fever, shock, and death if administered to humans or animals.

When the 1973 notice was published, available data and experience with LAL were not adequate to support its adoption as the final pyrogen test in place of the rabbit pyrogen test, which had been accepted and recognized for many years. To establish a database and gain experience with the use of LAL, that notice permitted the introduction of LAL into the marketplace without a license on the condition that its use be limited to the in-process testing of drugs and other products. The decision to use it was reached voluntarily by affected firms, and the labeling on LAL stated that the test was not suitable as a replacement for the rabbit pyrogen test.

Since that time, production techniques have been greatly improved and standardized so that they consistently yield LAL with an endotoxin sensitivity over 100 times greater than originally obtained. Moreover, it is widely recognized that the LAL test is faster, more economical, and requires a smaller volume of product than does the rabbit pyrogen test. In addition, the procedure is less labor intensive than the rabbit test, so it is possible to perform many tests in a single day.

In 1977 the FDA described conditions for the use of LAL as an end-product test for endotoxins in human biological products and medical devices (FDA 1977). The notice stated further that the application of LAL testing to human drug products would be the subject of a future *Federal Register* publication.

In 1980 FDA announced the availability of a draft guideline that set forth procedures for use of the LAL test as an end-product testing method for endotoxins in human and animal injectable drug products (FDA 1980). This draft guideline was made available to interested parties to permit manufacturers, especially those who had used the LAL test in parallel with the rabbit pyrogen test, to submit data that could be considered in the preparation of any final guideline.

The Guideline

The guideline (U.S. Department of Health and Human Services 1987) sets forth acceptable conditions for use of the *Limulus* amebocyte lysate test. It also describes procedures for using this methodology as an end-product endotoxin test for human injectable drugs (including biological products), animal injectable drugs, and medical devices. The procedures may be used in lieu of the rabbit pyrogen test.

Legal Effect

This 1987 guideline was issued under section 10.90(b) (21 CFR 10.90[b]) of the FDA's administrative regulations, which provides for use of guidelines to outline procedures or standards of general applicability that are acceptable to the FDA for a subject matter within its statutory authority. Although guidelines are not legal requirements, a person who follows an agency guideline may be assured that the procedures or standards will be acceptable to the FDA. The following guideline has been developed to inform manufacturers of human drugs (including biologicals), animal drugs, and medical devices of procedures that the FDA considers necessary to validate the use of LAL as an end-product endotoxin test. A manufacturer who adheres to the guideline would be considered in compliance with relevant provisions of the applicable FDA Current Good Manufacturing Practice (cGMP) regulations for drugs and devices and other applicable requirements. As provided in 21 CFR 10.90(b), persons who use methods and techniques not provided in the guideline should be able to assure, through validation, that the method or technique they use is adequate to detect the endotoxin limit for the product.

USP BACTERIAL ENDOTOXIN TEST AND RELATED MONOGRAPHS

The bacterial endotoxin test is a means of estimating the concentration of bacterial endotoxins present in or on the sample of the article(s) to which the test is applied. This test uses the LAL reagent obtained from the aqueous extracts of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus* (U.S. Pharmacopeia 1995).

The determination of the test end point is made with dilutions from the material under test in direct comparison with parallel dilutions of a reference endotoxin. Quantities of endotoxin are expressed in defined EUs.

Because amebocyte lysate has been formulated to be used for turbidimetric or colormetric readings, such tests may be used if shown to comply with the requirements for alternative methods. The tests require the establishment of a standard regression curve, and the endotoxin content of the test material is determined by interpolation from the curve. The procedures include incubation, for a preselected time, of reacting endotoxin and control solutions with LAL reagent and reading of the spectrophotometric light absorbance at suitable wavelengths. In the case of the end-point turbidimetric procedure, the reading is made immediately at the end of the incubation period. In the kinetic assay (turbidimetric and colorimetric), the absorbance is measured throughout the reaction period, and rate values are determined from those readings. In the endpoint colorimetric procedure, the reaction is arrested at the end of the preselected time by the addition of enzyme-reaction-terminating agent prior to the readings.

Reference Standard and Control Standard Endotoxin

The USP Endotoxin Reference Standard has a defined potency of 10,000 USP EU per vial. It is prepared as follows: constitute the entire contents of 1 vial of the RSE with 5 ml of LAL reagent water (LRW), mix intermittently for 30 minutes, using a vortex mixer, and use this concentrate for making appropriate serial dilutions. Preserve the concentrate in a refrigerator, for making subsequent dilutions, for not more than 14 days. Mix vigorously, using a vortex mixer, for not less than 3 minutes before use. Mix each dilution for not less than 30 seconds before proceeding to make the next dilution. Do not store dilutions, because of loss of activity by adsorption, in the absence of supporting data to the contrary. A control standard endotoxin (CSE) is a preparation other than the RSE that has been standardized against the RSE. Each new lot of CSE is to be standardized prior to use in the test. Calibration of a CSE in terms of the RSE must be with the specific lot of LAL reagent and the test procedure with which it is to be used. Standardization of a CSE against the RSE using an LAL reagent for the gel-clot procedure may be effected by assaying a minimum of one vial of the CSE and one vial of the RSE, as directed under Test Procedure, but using 4 replicate reaction tubes at each level of the dilution series for the RSE and 4 replicate reaction tubes similarly for each vial or aliquot of the CSE. The antilog of the difference between the mean $\log_{10}$ end point of the CSE is the standardized potency of the CSE, which then is to be converted to and expressed in EUs per nanogram under stated drying conditions for the CSE, or in EUs per container, whichever is appropriate.

A suitable CSE has a potency of not less than 2 EU/ng and not more than 50 EU/ng.

Preparatory Testing

The following procedure is used for preparatory testing. Use an LAL reagent of confirmed label sensitivity. Treat any containers or utensils employed so as to destroy extraneous surface endotoxins that may be present. Heating in an oven at 250° C or above for sufficient time is one way to do this.

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens of the article, or of solutions, washings, or extracts thereof to which the test is to be applied do not of themselves inhibit or enhance the reaction or otherwise interfere with the test. Validation is accomplished by performing the Inhibition or Enhancement Test as described below. Appropriate negative controls are included. Validation must be repeated if the LAL reagent source or the method of manufacture or formulation of the article is changed.

Test for Confirmation of Labeled LAL Reagent Sensitivity

Confirm the labeled sensitivity using at least one vial of the LAL reagent lot. Prepare a series of twofold dilutions of the RSE (or CSE) to give concentrations of 2 , 1 , 0.5 ,, and 0.25 , where is the labeled sensitivity of the LAL reagent in EU/mL. Perform the test on the four standard concentrations in quadruplicate and include negative controls. The geometric mean end-point concentration (see Calculations and Interpretation) must be 0.5 , and 2.0 . Confirm the labeled sensitivity of each new lot of LAL reagent prior to use in the test.

Inhibition or Enhancement Test

Perform the test on aliquots of the specimen, or a dilution not to exceed the maximum valid dilution (MVD), in which there is no detectable endotoxin. Perform the test on the specimen without added endotoxin and with endotoxin added to give final concentrations of 2.0 , 1 , 0.5 , and 0.25 . Perform the test as directed under Test Procedure, but using not fewer than 4 replicate tubes for the untreated specimen and for each specimen to which endotoxin has been added. In parallel with the above, test in duplicate the same endotoxin concentrations in water and untreated negative controls. Calculate the geometric

mean end-point endotoxin concentration for the specimen as described under Calculations and Interpretation. The test is valid for the article if the geometric mean end-point concentration in the specimen is 0.5 , and 2.0 .

If the result obtained for the specimens to which endotoxin has been added is outside the specified limit, the article is unsuitable for the Bacterial Endotoxins Test.

Repeat the test for inhibition or enhancement after neutralization, inactivation, or removal of the interfering substances or after the specimen has been diluted by a factor not exceeding the MVD. Use a dilution, not to exceed the MVD, sufficient to overcome the inhibition or enhancement of the known added endotoxin, for subsequent assays of endotoxin in test specimens.

If endogenous endotoxin is detectable in the untreated specimens under the conditions of the test, the article is unsuitable for the Inhibition or Enhancement Test; it may be rendered suitable by removing the endotoxin present by ultrafiltration, or by appropriate dilution. Dilute the untreated specimen (as constituted, where applicable, for administration or use) to a level not exceeding the MVD, at which no endotoxin is detectable. Repeat the test for inhibition or enhancement, using the specimens at those dilutions.

Maximum Valid Dilution

The MVD is appropriate to injections or to solutions for parenteral administration in the form constituted or diluted for administration, or where applicable, to the amount of drug by weight if the volume of the dosage form for administration could be varied. Where the endotoxin limit concentration is specified in the individual monograph in terms of volume (in EU per milliliter), divide the limit by , which is the labeled sensitivity (in EU per milliliter) of the lysate employed in the assay, to obtain the MVD factor. Where the endotoxin limit concentration is specified in the individual monograph in terms of weight or of units of active drug (in EU per milligram or in EU per unit), multiply the limit by the concentration (in milligram per milliliter or in units per milliliter) of the drug in the solution tested or of the drug constituted according to the label instructions, whichever is applicable, and divide the product of the multiplication by , to obtain the MVD factor. The MVD factor so obtained is the limit dilution factor for the preparation for the test to be valid.

Test Procedure

In preparing for and applying the test, observe precautions in handling the specimens to avoid gross microbial contamination. To quantify the amount of endotoxin in a specimen, an assay is performed on decreasing concentrations of specimens prepared by serial dilution. Select dilutions so that they correspond to a geometric series in which each step is greater than the next by a constant ratio. Include negative and positive controls and a positive product control.

Use not fewer than 2 replicate reaction tubes at each level of the dilution series for each specimen under test. A standard endotoxin dilution series involving not fewer than 2 replicate reaction tubes is conducted in parallel. A set of standard endotoxin dilution series is included for each block of tubes, which may consist of several racks for incubation together, provided the environmental conditions within blocks are uniform.

Preparations

Because the form and amount per container of standard endotoxin and of LAL reagent may vary, constitution and/or dilution of contents should be directed in the labeling. The pH of the test mixture of the specimen and the LAL reagent is in the range 6.0 to 8.0 unless specifically directed otherwise in the individual monograph. The pH may be adjusted by the addition of sterile, endotoxin-free sodium hydroxide or hydrochloric acid or suitable buffers to the specimen prior to testing.

Procedure

Into 10×75 mm test tubes or single test vials, dispense the specified volumes of negative controls, standard endotoxin concentrations, specimens, and positive product controls. Positive product controls consist of the article, or of solution washing or extract thereof to which RSE, or a standardized CSE, has been added to give a concentration of 2 . Add appropriately constituted LAL reagent, unless single test vials are used. Mix the specimen-LAL reagent mixture, and place in an incubating device such as a water bath or heating block, accurately recording the time at which the tubes are so placed. Incubate each tube, undisturbed, for 60±2 minutes at 37±1 C°, and carefully remove it for observation. A positive reaction is characterized by the formation of a firm gel that remains when inverted through 180°. Record such a result as positive (+). A negative result is characterized by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (−). Handle the tubes with care, and avoid subjecting them to unwanted vibrations, or false negative observations may result. The test is invalid if the positive product control is negative or the endotoxin

standard does not show the end-point concentration to be within ±1 twofold dilutions from the label claim of sensitivity of the LAL reagent or if any negative control is positive. Proceed to Endotoxin Content Calculation to determine the amount of endotoxin present in the test specimen.

Calculation and Interpretation

Geometric Mean Calculation

The end point is the last positive test in a series of decreasing concentrations of endotoxin, specimen, or specimen to which endotoxin has been added. Record the end-point concentration, *E,* for each replicate series of dilutions. Determine the lot end-point concentration, *e,* and calculate the geometric mean end-point concentration by using the following formula:

geometric mean end-point concentration=antilog (*e/f*),

where *e* is the sum of the log end-point concentrations of the dilution series used and *f* is the number of replicates.

Endotoxin Content Calculation

Calculate the concentration of endotoxin (in units per milliliter, gram, or milligram) in or on the article under test. First, calculate the end-point concentration, *E,* for each of a series of dilutions by multiplying the reciprocal of each end-point dilution factor by , where is the labeled sensitivity expressed in EUs per milliliter of the lysate used in the test. The geometric end-point concentration of the article under test is thus the antilog of *e/f,* where *e* is the log of the end-point concentration, and *f* is the number of replicate reaction tubes read at the level for the specimen under test.

Interpretation

The article meets the requirements of the test if the concentration of endotoxin is not more than that specified in the individual monograph.

EUROPEAN AND JAPANESE PHARMACOPOEIA ACCEPTANCE OF THE LAL TEST

The European Pharmacopoeia (EP) closely followed the USP in describing an endotoxin test using LAL. Basically, the EP test is similar to the USP Bacterial Endotoxins Test, with the exception that the EP may place more emphasis on a pass or fail decision than on quantita tion. The EP Guidelines also state that the substitution of the LAL test where a rabbit pyrogen test is specified in the product monograph requires validation and the agreement of the competent authority. Pyrogenic batches, if available, should be tested with the LAL test as part of the validation. In any case, the gel-clot test is emphasized (as with the USP and Japanese Pharmacopoeia [JP] tests). The latest version of the EP Endotoxin Test (ET) and accompanying guidelines can be found in the 1998 EP Supplement (European Pharmacopoeia 1998). Apart from an incubation requirement of 37°C, the EP-ET is the most flexible with respect to gel-clot methodology. For example, the EP-ET allows choice of sample and reagent volumes (thus allowing micromethods, i.e., methods that employ lesser amounts of lysate than the "standard" assay) as well as Incubation time (20–60 minutes). Otherwise, the sample under test cannot be diluted past the MVD; a negative control must be included, as well as a 2 positive product and positive control. The EP Guidelines provide details on determination of MVD and endotoxin limits. In addition to the controls, samples must be replicated. A retest is allowed only if one replicate of the sample passes and the other fails. A detailed review in a comparative format (USP vs. EP vs. JP) is provided by Dawson (1997b).

The Japanese Pharmacopoeia also closely follows the USP and EP with respect to LAL, although the JP pays more attention to the "photometric methods," perhaps because they are more widely used in Japan than in the United States and Europe. Like the EP, the JP allows use of lysates from either *Limulus* (LAL) or *Tachypleus* (TAL). For the gel-clot method, the JP is quite specific, but very similar to the USP; i.e., 37+1°C, 60±2 minutes, negative, positive, and positive product controls, and so forth. Retest is similar to that specified by the EP; i.e., one is allowed if one replicate is negative and the other positive. Although there is a Japanese reference endotoxin standard, the endotoxin units used in the JP are in terms of the USP RSE, to which the JP endotoxin was compared.

International Standard

As should already be apparent, many endotoxin standards have been used over the period of time the LAL test has been gaining acceptance. It is very likely that too many standards prevented adequate comparison of LAL in some of collaborative studies. In many cases, the LAL reagent(s) were blamed instead of the standards. Pharmacopeial jealousy and national pride also played a role in slowing the acceptance of the LAL test, by insistance on "official" standards. At one point, the U.S. FDA and USP, the EP, the WHO, and the JP each had its own "reference standard." Unlike a platinum meter stick or an atomic clock, endotoxin is a biochemical subject to influences of drying, ions, pH, detergents, and so forth. Thus collaborative studies comparing one official reference to another became statistical nightmares in which the users, often given only the "average" unitage of the standard, could not understand why their LAL did not perform according to label claim. The World Health Organization, under the guidance of the NIBSC, teamed with the USP and the FDA to manufacture and test a new international standard (IS) (Poole et al. 1997). This standard, designated EC-6 by the FDA, Lot G by the USP, the second IS (preparation 94/580) by the WHO, and BRP-3 by the EP, is now accepted by these organizations. The JP has indicated it will maintain its own reference standard.

The common standard and acceptance of the EU goes a long way toward harmonization. Although the pharmacopoeias are not entirely in agreement, the gel-clot methodology is close enough for comfort, and true pharmacopeial harmonization for the LAL test may be within sight.

METHODOLOGY

Gel-Clot Assay

The gel-clot assay is the simplest LAL method. The basic test protocol requires mixing 0.1 mL of sample with 0.1 mL of reconstituted LAL in a 10×75 mm test tube, mixing gently, and incubating undisturbed in a 37°C water bath or dry block for 60 minutes. To read the test, the tube is gently inverted. If a gel is formed that can withstand an inversion of 180°, the test is positive. Anything less is negative. Particular details of the test can be found in the manufacturer's instructions and in the various pharmacopeias. It should be noted that the manufacturer's instructions take precedence and should be followed exactly for expected results. Reagent storage, reconstitution, and glassware or plastic ware used with the assay can all influence the test.

Turbidimetric Assay

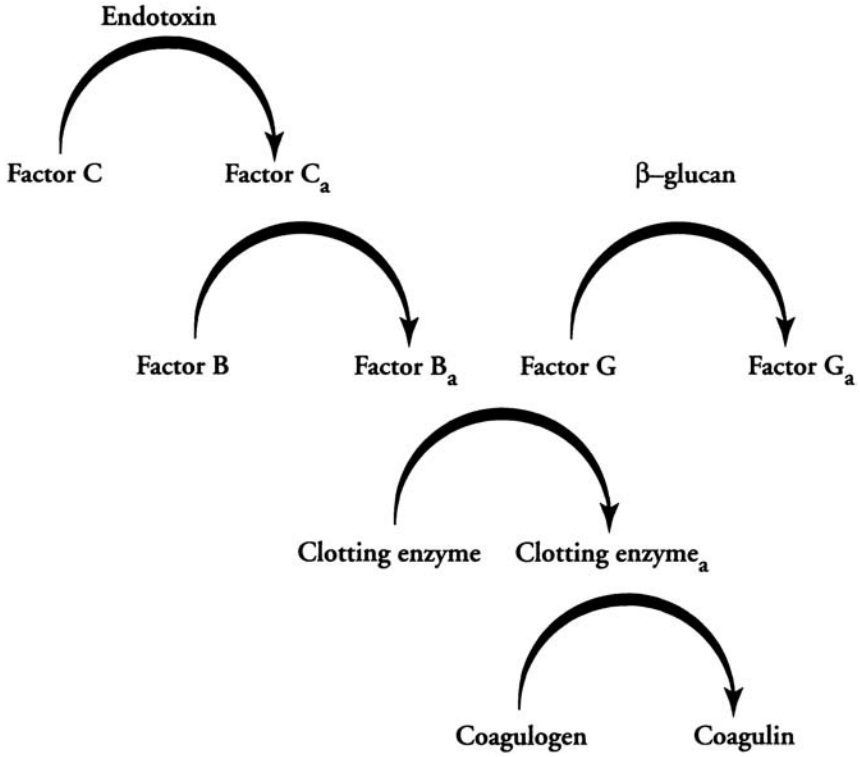
The turbidimetric assay relies on the fact that the protein formed in the development of a gel clot also forms a turbid solution. The turbidimetric assay can therefore be viewed as an objective way of following clot formation. However, degree of turbidity and the formation of a solid gel clot are not tightly correlated, although attempts have been made to relate the two so that certain machines can be accepted as "gel-clot readers." The ability of the turbidimetric method to accurately measure endotoxin however has been adequately demonstrated. Likewise, correlations among the turbidimetric, chromogenic, and gel-clot methods have been shown to be excellent within the error of the methodologies.

For the turbidimetric assay, 0.1 mL of LAL is mixed with 0.4 or 0.1 mL of sample in a tube (e.g., for assays employing the LAL-5000 or Toxinometer) or microplate (e.g., for assays employing the KQCL) and placed in the machine (or incubator for the end-point method). For the kinetic method, incubation varies but since reading is continuous or semicontinuous, only a maximum time must be determined. For the end-point method, a maximum time must be determined from experiment, and subsequent readings must be made precisely on time. Most readers now can be coupled with computer programs to collect and analyze data. Most programs perform a linear regression, using the time it takes to reach a certain optical density or percentage transmission and the endotoxin concentration. At least one program uses a curve-fitting algorithm because some lots of LAL do not show a precisely linear response when a spectrophotometer is used to measure turbidity. Non-curve-fitting programs avoid this problem by selecting a 1 or 2 log range of standards rather than trying to force a 4 or 5 log range on the assay. Although the original kinetic assay was developed to allow for a range of sensitivities (gel clot is set at one concentration, while end-point tests are limited to a one log range), for pharmaceutical applications where a one concentration limit or spike control is used, greater precision can be achieved and time saved by using a smaller standard range. Incubation of the turbidimetric test is also 37°C, only the time of incubation varying.

Chromogenic Assay

The chromogenic assay is basically a turbidimetric assay with an added chromogenic substrate. These substrates mimic the coagulogen component of the assay and release a chromophore, paranitroaniline (pNA), when cleaved by activated

Figure 11.1 *Limulus* amebocyte lysate cascade.



From LAL Update, September 1995, Vol. 13, No. 3. (Reprinted with permission of Associates of Cape Cod, Inc.)

proclotting enzyme (see Figure 11.1) The chromogenic assay can be performed as an end point, end point with diazo coupling, or kinetic, depending on the reagent or test kit. The end-point test is performed two ways, depending on the kit. If the reagent is supplied as a combined LAL-substrate, the reconstituted LAL, 0.05 or 0.1 mL is added to 0.05 or 0.1 mL of sample in a tube or microplate and incubated at 37°C for a period of time. At the end of incubation, the sample is either read directly or the reaction is stopped with the addition of acid and read later. In the case of the diazo-coupled end point, the reaction is converted to the diazo derivative with the chemistry provided with the kit and then read. For pNA, a spectrophotometer or plate reader equipped with a 405 nm filter is required. For the diazo-coupled reaction, 540–550 nm is required. Some older kits supply the chromogenic substrate separate from the LAL. For these end-point tests, a primary incubation is performed with the LAL-sample followed by addition of the substrate and a short secondary incubation, all at 37°C. After the second incubation, the reaction is read immediately or stopped with acid and read later. For the kinetic test, the sample and LAL are mixed and the reaction placed immediately in an incubating plate or tube reader. Data collection for the kinetic chromogenic test is similar to that for the kinetic turbidimetric test.

AUTOMATION

The kinetic turbidimetric and chromogenic tests described above have been considered "automated" in that the operator need only load the test and walk away. The computer, in concert with the plate or tube reader, times and incubates the test and collects and analyzes the data. With these methods, a user could load a test prior to leaving for the day and retrieve the results the following morning or a week later.

The turbidimetric and chromogenic tests both lend themselves to robotics—i.e., eliminate the operator in the preparation of samples and standards, and in loading the machine. The LAL-5000 has been successfully coupled to a Zymate robot and the KQCL has been hooked up to a Beckman system. These methods, however, lend themselves only to very large operations and require extensive validation and attention to detail due to short reconstituted shelf life of lysate, propensity of standards to adsorb to the walls of containers and tubing, and the ubiquitous nature of bacteria and endotoxin in the environment (facilitating contamination).

RESEARCH AND CLINICAL APPLICATIONS

It was always hoped that the LAL test would prove useful as a clinical diagnostic tool. Studies in the literature indicated that the LAL test could detect gram-negative spinal meningitis and urinary tract infections. A test for gonorrhea was also proposed. LAL manufacturers were actually approved to market tests for spinal meningitis and gonorrhea but never did. Although the LAL test is used as a diagnostic in Japan, a clinical trial for an LAL as an aid in the diagnosis of gram-negative sepsis was done in the United States, but the results did not indicate clinical utility (Ketchum et al. 1997). In light of additional findings that antiendotoxin therapies are not efficacious in reducing mortality or morbidity in septic patients, it is surprising that the LAL test is still considered by some to hold diagnostic promise for this condition. One reason given for the lack of clinical utility of the LAL test is its exceptional sensitivity to endotoxin. The many interfering factors present in clinical samples are thought to mask the ability of LAL to adequately measure endotoxin. In addition, especially in blood, endotoxin can occur in the absence of bacteria, and too few bacteria may be present to cause a positive LAL test. It appears unlikely that a clinical application for LAL will appear since first proposed almost 30 years ago. Excellent reviews of clinical applications have been published (Novitsky 1996; Prior 1990).

LAL as a Research Tool

The LAL test has been used extensively as a research tool to follow the fate of endotoxin in animal models, environmental systems, water, and food processing. As a tool for the detection of endotoxin, the LAL test is unsurpassed; no other test comes close to its sensitivity and specificity. For reviews see Watson et al. (1982, 1987) and Pearson (1985).

FUTURE OF THE ASSAY

We are amazed that the LAL assay is still the dominant, indeed almost the only practical, test for endotoxin. However, there are indications that the horseshoe crab's very existence may be threatened due to overharvesting the animal for use as eel and whelk bait (Kerlinger 1998). Ever since the LAL test was first described, scientists have endeavored to elucidate the reaction components. These have now been thoroughly described. Not surprisingly, recombinant analogues of at least factor C have been developed and have been cloned in yeast (Ding 1997) and in insect cells (Ding and Ho 2002). A chromogenic substrate for the coagulin component has been in use for more than 15 years. In one of our laboratories (TN), an endotoxin binding protein (rENP) from *Limulus* has been cloned and has been developed into an assay (Novitsky et al. 2001). This new assay utilizes the principle of fluorescence polarization to detect and quantify endotoxin bound to fluorescently labeled rENP. It is very fast (10 seconds) and easy to perform. However, the sensitivity of the current reagent and reader is lower than for LAL. Should research succeed in producing an adequate sensitivity with either a true recombinant LAL replacement or a completely new endotoxin assay, given the history of acceptance of the LAL test, it may be a while before an "official" replacement assay appears, however exciting the science may be.

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12
A Practical Guide to Validation

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The adoption of a new, rapid microbiological method has many advantages. Increased accuracy and precision are often associated with instrument-based methods, because instruments reduce operator error and provide automation. In addition, rapid methods often offer huge financial savings to an organization. However, the enthusiasm to implement a new method can come to an abrupt end when the dreaded validation word looms large. This chapter is designed as a simple guide to bringing any of the exciting new methods described elsewhere in this book onstream in your company. It may be overly simplistic to those with experience in validation as it is aimed at those trying to change a microbiology method for the first time.

There are two common reactions to validation: paralysis and overzealousness. Overvalidation by some gives validation a reputation of being too much work, leading in turn to greater paralysis that benefits no one. What follows clearly lays out the stages in microbiological validation, indicating the help that suppliers can be expected to provide and suggesting where within your own organization advice may be sought. Validation efforts should not prevent you from delivering to your organization all the benefits of rapid methods, if you keep in mind throughout the single objective of proving that the method is fit for the purpose.

WHAT IS VALIDATION?

Validation is an often-misused word, so it is useful to start from the basics. In a regulated environment, validation is the evidence provided to a third party to prove that a method is suitable for its intended use and capable of producing data that are consistent and reliable.

Validation is the demonstration that a test method will perform as expected, that it will consistently meet requirements under both ideal and challenged conditions, in the user's laboratory, in the hands of the intended user. Therefore the validation study must be conducted where the method will be routinely performed. In addition the validation study should include the use of "real" samples using standard sample storage conditions before testing.

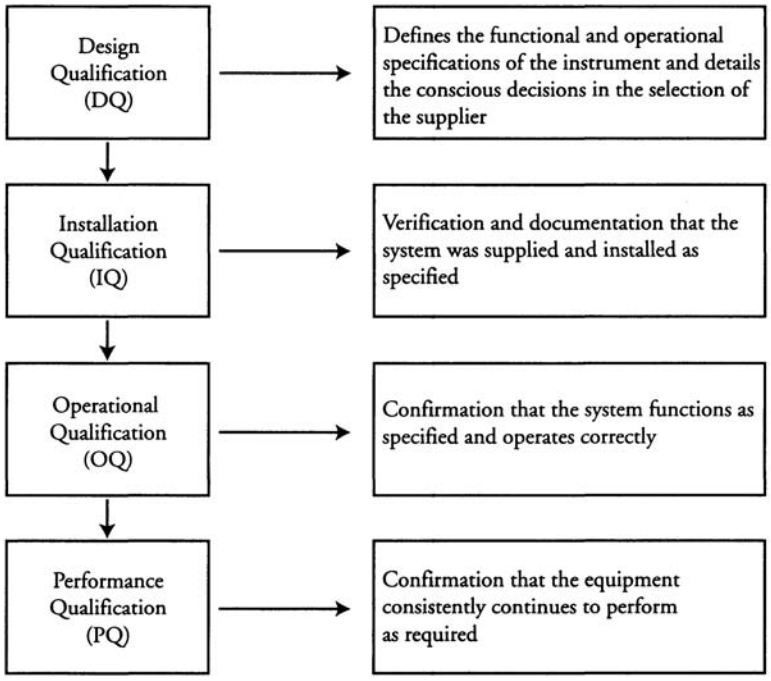
The user has ultimate responsibility for ensuring that a new method is adequately validated and can be defended by the organization during a regulatory inspection.

BACKGROUND

The International Conference on Harmonisation (ICH) put together the guidelines "Validation of Analytical Procedures" in 1994. Since that time these have been incorporated into the United States, European, and Japanese Pharmacopoeias. Although these guidelines are the only official recommendation on how to validate an analytical procedure, they really cover only testing for impurities and active moieties in drug substances. The general principles described are, of course, applicable to methods in general, but do provide some practical obstacles when trying to validate a microbiological method.

Equipment qualification (EQ) is well described for validating process equipment and computer systems, but less so for laboratory equipment. Article 8 of EEC (European Economic Community) Directive 91/356/EEC on good manufacturing practice, published by the Commission of the European Communities, states, "…equipment…critical for the quality of the products shall be subjected to appropriate qualification." ISO-9001 section 4.11 (International Standards Organization) states that "the user shall identify, calibrate and adjust all injection, measuring and test equipment and devices that can affect product quality at prescribed intervals." EQ is divided into four stages, outlined in Figure 12.1. See Freeman et al. (1995) and Valid Analytical Measurement Working Group (1996) and www.gamp.org for a more detailed review.

Figure 12.1 The equipment qualification process.



The validation criteria (accuracy, precision, specificity, limit of detection, limit of quantitation, linearity, range, ruggedness, and robustness) are defined by ICH (1994a, 1994b) and in the USP (U.S. Pharmacopeia) chapter 1225. These definitions take no account of microbiology; proposed redefinitions were put forward by Wills et al. (1998) and further developed and incorporated in the Parenteral Drug Association (PDA) Technical Report No. 33 (TR-33, 2000). A summary of the validation definitions for microbiology is shown in Table 12.1.

TR-33 also included guidance on determination and proposed acceptance criteria. In addition equivalence was included. These redefinitions are under consideration by the USP microbiology subcommittee. At the time of writing, a draft chapter 1223, "Validation of Alternative Microbiological Methods" has been published for comment (USP 2002). See TR-33 for further help in validating alternative microbiological methods.

START AT THE BEGINNING

The whole process of validation starts from the moment when you determine that the current method could be improved. By inadvertently, and probably informally at first, defining the need for a new test and looking in the marketplace for an alternative, you get the first part of validation underway. Once some of the initial thoughts have started to gel, you may find it useful to start writing them down in a first draft of the Specification Qualification.

SPECIFICATION QUALIFICATION (SQ)

Specification Qualification is the term for the first phase of validation. Put simply, it is the documenting of those questions (and answers) that you will automatically ask when you start considering a new method.

- What samples must the test be able to analyze?
- What range of sample types should the test method accommodate?

Table 12.1 Definitions of Method Validation Criteria for Microbiological Testing

Validation Parameter	Definition
Accuracy	Closeness of the results to the compendial method
Precision	Degree of agreement among individual test results

Validation Parameter	Definition
Specificity	Ability to detect and/or enumerate a specific organism or class of organisms from a particular sample matrix
Linearity	Ability to count organisms that are directly proportional to the numbers in the sample
Range	The upper and lower microbial count that has been demonstrated to provide accurate and precise counts
Limit of Detection	The lowest microbial level that can be detected but not necessarily quantified
Limit of Quantification	The lowest microbial level that can be reasonably quantified
Ruggedness	The degree of reproducibility of microbial counts with respect to test conditions; e.g., different equipment, different operators, different labs, different days
Robustness	Capacity of the method to remain unaffected by small deliberate variations in media, reagents, incubation conditions, and so forth

- In what length of time must the test be completed?
- What accuracy is required?
- What sensitivity, selectivity, limit of quantitation, and limit of detection are required?
- What precision is required; over what range of values?
- What types of interfering substances are likely to occur; will they affect accuracy or precision of the test?
- What method must the results of the new method correlate with?
- Where is ruggedness important?

When it starts to become clear what the new method should be able to do and what methods are available commercially, you will also need to consider your requirements of the suppliers of such methods. These typically include:

- Demonstration of compliance with an appropriate quality system;
- Employment of suitably qualified and experienced staff throughout development, manufacture, installation, and maintenance;
- Provision of a system for change control and system updates;
- Provision of a certificate of performance against declared specifications;
- Provision of a definition of on-site and telephone support available;
- Provision of clear, easy-to-use manuals, identified by version number;
- Provision of certificates of safety;
- Third party accreditation of test methods;
- Logging of a Drug Master File (DMF) with the U.S. Food and Drug Administration (FDA);
- Publication of the technology, on which the test method is based, in peer-reviewed journals;
- Provision of support with regulatory acceptance; and
- Education of the regulator with respect to this technology or test method.

Furthermore you may wish to consider the regulatory and economic implications to your company that changing a method would entail. This is the time to start talking with others in your organization, gathering an informal validation team, and preparing a draft validation plan.

VALIDATION PLAN

This is a summary document, detailing the overall intention and approach to establishing that the new test method has adequate performance. It may cover the following areas:

- Goal of the study;
- Study objectives in measurable terms;
- Definitions of any unusual terminology;
- Pre-study requirements;
- Study configuration—details of the stages and supporting documentation necessary;
- Sample requirements—what, how much;
- Test parameters and methods;
- Test acceptance criteria;
- Testing schedule;

- Sampling requirements—sampling techniques, sampling locations;
- Data handling;
- Reporting requirements; and
- Study acceptance criteria.

The validation plan should be regarded as the overview document, referring to the individual sections of the qualification process for the detailed description. It should also identify the validation team responsible for ensuring that the new method is implemented swiftly but correctly. The validation team may comprise the microbiologist responsible for performing the testing and a senior representative from the Quality Assurance staff, someone from compliance with validation experience in other parts of the organization, and perhaps someone from manufacturing. Each stage of the validation process should be reviewed and given the appropriate authorization before the process continues.

DESIGN QUALIFICATION (DQ)

Design Qualification is the second stage of validation, in which the requirements defined within SQ are compared to a specific test method from a certain supplier. The supplier should be able to provide data and information to prove that the test method is fit for the purpose and to show the supplier is suitable to become a pharmaceutical supplier. It is likely that a compromise between the ideal requirements of the user and those that are practically deliverable by a commercially available test method will have to be reached. This should also be documented.

It is the responsibility of the user to ensure that DQ is appropriate and is correctly documented, although it is likely to be prepared jointly by the supplier and the user.

A key objective throughout validation is to keep the amount of work to a minimum. That should be borne in mind when assessing supplier data and deciding what practical validation work should be done. Some confidence should be placed in the large amount of supporting data each product has and a critical eye used to discern which are the critical elements to be repeated on site. A supplier's data indicating that a large number of microorganisms have been detected in a large number of products may translate to a user validation protocol involving verification that the test method works for a few critical microorganisms in the user's specific products.

A validation protocol can be defined at this time, incorporating installation, operation, and performance qualification.

INSTALLATION QUALIFICATION (IQ)

Installation will often be carried out by the supplier and witnessed throughout by the user. The supplier should be able to provide a comprehensive protocol and be open to its being incorporated into company style.

The IQ protocol includes the following steps:

- Unpack the equipment and check against the order.
- Record serial numbers.
- Ensure that adequate safety precautions are in place.
- Site the equipment correctly.
- Power-up the equipment.
- Perform essential firmware verification checks
- Install testing protocols.
- Perform key test to verify the performance of the equipment supplied.

The equipment is now up and running in your facility. The visit by the supplier should also involve comprehensive training of anyone who is going to be involved in the validation. It is important that the installation is carefully scheduled such that the trained persons are free to start work whilst that training is fresh in their minds.

OPERATION QUALIFICATION (OQ)

This section forms the main practical part of validation, verifying that the new test method performs in your laboratory on your products in a way similar to that claimed by the supplier. Operation qualification should be tackled in manageable sections, perhaps in a phased approach in which groups of five products head toward release by the new test method.

The first section may be viewed as a confirmation of proof of principle, should one be required. It consists of testing of five products to prove that they are compatible with the technology and perhaps proving detection of low numbers of a single microorganism.

This phase may be performed prior to large amounts of the documentation being put in place, thus preventing possible wasted effort. When a decision is made to commit to the validation of the new test method, the data generated are still valid if performed on the user site with a system conforming to the IQ part of the protocol.

The second phase can be termed verification, in which critical parts of the supplier's supporting data are repeated to show that the test method works in your laboratory with your products. Base the work around the validation criteria, but include reference to as much of the supporting data as possible. You may, for example, choose a limited panel of microorganisms, including a couple of isolates from your plant. You may include the testing of some naturally contaminated products.

You judge how much data needs to be generated, but remember the perils of overvalidation. A balance should be struck between getting the new method into routine use and demonstrating that it is fit for the purpose.

PERFORMANCE QUALIFICATION (PQ)

This provides the confirmation that the system consistently continues to perform as required. It refers to the daily positive and negative controls that should be run to monitor system performance and to the daily, weekly, and monthly maintenance checks that should be carried out. PQ also documents the service and calibration performed by service engineers, training records of operators of the system, and standard operating procedures pertaining to the system (see below).

PQ is in fact no more that a protocol with record forms, which will be carried throughout the lifetime of the test method

STANDARD OPERATING PROCEDURES (SOPs)

Standard Operating Procedures written in your company style should cover each aspect of the operation and maintenance of the new test method. The need for effective instructions is of utmost importance because personnel need to understand exactly what to do. SOPs should be appropriate, clear, accurate, and approved by appropriate individuals.

Often the supplier will provide the instructions for the system electronically so that they can be incorporated into your internal SOPs with minimum effort.

VALIDATION SUMMARY

Each phase of validation should be reviewed and approved before you move on to the next. One of the most critical review points, however, comes with the preparation of the Validation Summary. This provides a synopsis of the validation objectives and data, showing that the acceptance criteria have been met and making the recommendation that the test method should be implemented.

IMPLEMENTATION

Following approval to move on, the final phase is running the new test method in parallel with the existing method. At first, this means releasing the product only following the results of the existing method, but comparing the results to the new method. As confidence in the new method increases, perhaps following a pre-specified period of time or number of production batches showing equivalence between the two methods, the switch can be made to releasing product from the results of the new method. Initially the old method may continue to be run in parallel, as insurance, but following a prespecified period of equivalence the old method should be dropped.

Prior to the switch to the new method, a review of the final phase of validation should be performed and the change authorized. Some products may require that to release a product, license amendment will need to be sought. In other instances, the new method will have to be justified to a regulator at inspection.

Whatever level of regulatory approval is required, the documentation defined above should stand you in good stead. Comments to guide you through the product license amendment process are provided under Amendments to Product Marketing Authorizations, below.

PERIODIC REVIEW

When a validated method is used in a regulatory environment, validation does not end with the approval to use the method. When the test method is in routine use, a formal mechanism should be put in to place to review its performance periodically. This can be as simple as running the old method periodically (for example, once every year) to verify that the new method is still performing.

MULTIPLE SITE VALIDATION

It should be possible to define a much more limited validation study to transfer a validated rapid microbiological method from one manufacturing site to another within the same organization. Assess the critical elements that vary site-to-site and incorporate into the validation protocol elements to determine that these variables do not affect the performance of the test. Installation qualification should be performed at each site by the supplier to verify that the system is delivered and installed to specification. The products to be tested may vary from site to site, and so a limited study should be performed to verify the performance of the method. Other parts of the validation are likely to differ very little.

You should assess whether the entire validation documentation needs to be held on each site or whether the master validation documentation can be referred to. It is essential to remember that each site will be required to justify the use of the rapid test method to an inspector. If the individuals are not sufficiently familiar with the validation, then support may be required.

AMENDMENTS TO PRODUCT MARKETING AUTHORIZATIONS

The regulators, like the rest of us, have too much paperwork and are unlikely to be impressed when they receive a copy of your entire validation documentation in support of an application to amend a product license. The recommendation is to prepare a short summary document in the style of a scientific paper.

Clearly state the reasons for wanting to change the method, describe the new method, and present the data to support the change. Key aspects of improvement in product safety should be highlighted—for example, the new method is more accurate or more precise.

Any products that are licensed for sale in the United States are governed by the FDA. FDA guidance on obtaining changes to approved NDAs (New Drug Applications) describes different reporting categories.

Any change in a regulatory analytical procedure, such as the inclusion of a new microbiological method, could be described as a moderate change. In this case a Supplement—Changes Being Effected in 30 Days document—can be filed. The applicant must submit information that assesses the effect of the change and demonstrates the equivalence of the product before and after the change.

The Changes Being Effected in 30 Days route has not been used by manufacturers wanting to use a rapid method; some manufacturers have argued that this is a minor change and have included a description in their annual report.

The guidance document that will help you to assess what type of supplement to file can be located at www.fda.gov/cder/guidance/2766fnl.htm.

The European Union has adopted a system of variations to marketing authorizations. Such variations are described as common practice, and they may be introduced to take account of technical and scientific progress, add safeguards, or reflect evolving therapeutic indications. There are two types of variation to a marketing authorization: Type I and Type II. Type I variations have to be granted or otherwise within 30 days of application and include Changes to Test Procedures of the Medicinal Product. There is an application form to be completed and supported by emended documentation and supporting information.

This Type I amendment application has been used successfully on many occasions by manufacturers to gain approval for the use of the Celsis RapiScreen™ test.

CONCLUSION

It is possible to change a microbiology release method for a licensed product; it has been done on numerous occasions in many countries. Validation will always be a time-consuming process, but with clear guidelines and a focus on the key issues it can be relatively painless.

Good documentation is the key to validation; doing it correctly can save time in the long run. Many of the stages described in this chapter can be simply documented in very few pages. In some cases, summary information may be held by the user, or detailed information that is available for regulatory inspection may be held in a DMF or Claim Support Files held by the supplier. Much of the other information required may be provided electronically by the supplier. Your organization is likely to have standard formats into which this product-specific information can be pasted.

It is important to demonstrate good science throughout the validation and to show that the new method is equivalent to or better than the old method. Considering the limitations of traditional methods, many of which are outmoded and cannot satisfy the tighter analytical requirements of modern manufacturing, this should not be too difficult.

The two key things to bear in mind throughout validation are:

- What you are trying to show—that the new method is fit for purpose; and

- Why you are doing it—everything from relieving the work load in the laboratory to saving your company significant amounts of money.

A focus on these should ensure that you don't get dragged into overvalidation and excessive documentation and will mean that the benefits of the new method are swiftly and efficiently delivered.

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13
Regulatory Recognition and Acceptance

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OBJECTIVES

Regulators are expected to evaluate a wide range of manufacturing and test methods as part of their responsibility to safeguard the public health. Concerns of regulatory authorities with respect to changes in test methods in the pharmaceutical industry include the scientific validation of the new methods and correlation of their results with the results from standard or "classical" methods. These concerns become very difficult to weigh objectively in the area of microbiological tests because of the technical variability inherent in some of these tests and the high level of variability in sampling. This issue is addressed more completely in Chapter 12, (on validation), and it is the subject of PDA (Parenteral Drug Association) Technical Report 33, "Evaluation, Validation and Implementation of New Microbiological Testing Methods," May/June 2000. Because most of these assays are related to purity, potency, or safety of pharmaceutical products, regulatory agencies view them as critical. Agencies require scientific evidence in support of validation and use of these methods.

This chapter is written from my perspective as a former review scientist and inspector for the Center for Biologics Evaluation and Research (CBER) of the U.S. Food and Drug Administration (FDA) and as a current member of the Analytical Microbiology Expert Committee of the U.S. Pharmacopeia (USP) and the PDA Task Force for the PDA Technical Report 33, mentioned above. I include here the views of other groups, e.g., European Union (EU), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP), and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) as much as possible, but concentrate on FDA and USP concerns.

BRIEF HISTORY OF REGULATION DEVELOPMENT

Like all national regulatory authorities, the U.S. FDA is empowered by federal laws, specifically the Food, Drug and Cosmetic (FDC) Act (1938), to protect the public from harmful or adulterated products as specified under the law. This law states, in part, "A drug or device shall be deemed to be adulterated…(b) If it purports to be or is represented as a drug the name of which is recognized in an official compendium, and its strength differs from, or its quality or purity falls below, the standards set forth in such compendium. Such determination as to strength, quality, or purity shall be made in accordance with the tests or methods of assay set forth in such compendium…." The USP is recognized as the official compendium to which this law refers, and its test methods are considered validated by the FDA.

Biological products were regulated under a separate U.S. law (Public Health Service Act of 1912) for many years, but after the transfer of biologics regulation to the FDA in 1972, applicable sections of the FDC Act have been applied more often to these products. Recent guidance documents from CBER and the Center for Drug Evaluation and Research (CDER) indicate that this trend will continue in the future.

Some of the most important regulatory documents from the FDA are the Current Good Manufacturing Practice (CGMP) regulations. Originally promulgated in 1963, these regulations have been amended several times, the most recent amendment having been proposed in 1996. One part of the CGMP regulations, 21 CFR (U.S. Code of Federal Regulations) 211.160 (b) requires that "…drug products conform to appropriate standards of identity, strength, quality, and purity." This is considered central to the question of the use of new test methods in the pharmaceutical industry, because these test methods will, at least in part, determine these characteristics. In the science of microbiology, the appropriateness of a test is often subjective and not easily defined.

Other testing regulations that apply to pharmaceutical products include filing requirements for technical sections found in 21 CFR 314.50(d)(1), Chemistry, manufacturing and controls; 21 CFR 314.70, Supplements and other changes to an approved application (for drugs); 21 CFR 601.12, Changes to an approved application (for biologics) and 21 CFR 430, Antibiotic Drugs. The first three of these require descriptions of testing methods used in manufacturing controls and for product release tests. The last provides specific test methods. These and other regulations are in the process of change. Under the Food and Drug Administration Modernization Act of 1997 (FDAMA), the FDA is required to streamline filing requirements and to provide guidance that will assist the industry in the implementation of changes to applications. FDAMA will affect the regulation of products by CDER, CBER, the Center for Devices and Radiological Health (CDRH), and the Center for Food Safety and Applied Nutrition (CFSAN).

When the FDA wishes to change an existing regulation or propose a new one, it publishes a Notice of Proposed Rulemaking in the *Federal Register* for comment by the public. After a suitable comment period (e.g., 90–180 days), all comments are considered by the agency, and the notice is either republished for further comment (if substantial changes were made) or published as a Final Notice with an effective date for the regulation. Because this is such an intensive and time-consuming task, the FDA will often publish Guidance Documents or Points To Consider so that industry may understand the thinking of the agency on particular topics and have a chance to comment on them in a more timely fash ion. These Guidance Documents are not legal requirements but are issued under the procedural provisions of U.S. law (21 CFR Part 10.90) and are of general applicability to the CGMP regulations. Although these documents are not legally binding on either the FDA or industry, manufacturers may rely on them with the assurance of their acceptability to the FDA. Industry may also follow different procedures as long as they are fully validated. The FDA also publishes Inspection Guides so both agency inspectors and industry can understand what will be required during an FDA inspection. Two of these guides are discussed in more detail later in this chapter.

The USP standards are used by the FDA to determine the identity, strength, quality, and purity of pharmaceutical articles. The General Chapters section of the USP (Chapters 1–999) includes mandatory requirements for tests and assays. Any proposed change in an existing test or a new test proposal must first be published for comment in *Pharmacopeial Forum, (PF),* the USP Journal of Standards Development and Revision. The article, including all supporting data, would be reviewed by the appropriate USP Expert Committee prior to publication in *PF*. In the case of our topic, the Analytical Microbiology Expert Committee would perform this function. Subsequently, all comments on the article would be sent to that committee and shared with the authors. A revised article would then be published as an In-Process Revision in *PF* for further comment as needed until the committee is satisfied that all substantive issues have been resolved. The final step would be acceptance by the USP as indicated by publication in the next edition of the *Pharmacopeia* or in a supplement to the current edition.

The ICH was organized to provide an opportunity for harmonization of technical requirements for the registration of pharmaceutical products among three regions: the European Union, Japan, and the United States. Harmonization initiatives are developed with input from both regulatory and industry representatives. In addition, the International Federation of Pharmaceutical Manufacturers Associations (IFPMA) participates as an umbrella organization for the pharmaceutical industry and provides the ICH Secretariat to coordinate the preparation of documents. The ICH Steering Committee (two members from each of the seven parties to ICH) oversees the work, which is carried out to prepare for the International Conferences on Harmonization and to follow up on recommendations from these conferences. Three Expert Working Groups (EWGs) have been set up to advise the Steering Committee on technical issues concerning safety, quality, and efficacy. The Steering Committee selects topics to proceed through the ICH process of preparing a draft guideline and consulting with regulatory and industry members. After a series of successive steps of review and acceptance within ICH, a final draft is submitted to the Steering Committee, which recommends it to the three regional regulatory bodies for adoption. Each regulatory authority then takes the necessary administrative action to adopt the recommendation according to its own internal procedures. In the United States, the FDA publishes the guideline in the *Federal Register.*

There are two ICH documents listed under the Quality topic that are relevant to this chapter and have reached the final stage of acceptance. These are Guideline Q2A: Validation of Analytical Procedures—Definitions and Terminology (FDA 1995) and Guideline Q2B: Validation of Analytical Procedures—Methodology (FDA 1997a). Guideline Q2A identifies the validation parameters needed for a variety of analytical methods. It also discusses the characteristics that must be considered during the validation of the analytical procedures that are included as part of registration applications. Guideline Q2B extends the above text to include the actual experimental data required, along with the statistical interpretation, for the validation of analytical procedures.

REGULATORY CONCERNS

Inspections of manufacturing establishments by representatives of regulatory agencies all tend to concentrate in areas of compliance with applicable Good Manufacturing Practice regulations. In the United States these are found in the Code of Federal Regulations (CFR) at 21 CFR, Parts 210 and 211 (Code of Federal Regulations 1997). The FDA also published a

Proposed Rule (FDA 1996) amending and clarifying certain parts of the cGMP regulations. Some examples of sections of these regulations that would have an impact on recognition and acceptance of alternative test methods are the following.

- 211.22(c)—The quality control unit has the responsibility for approving or rejecting all procedures or specifications impacting on the identity, strength, quality, and purity of the drug product.
- 211.84(d)(6)—Each lot of a component that is liable to microbiological contamination shall be subjected to microbiological tests before use.
- 211.94 (d)—Standards, specifications, and methods of testing shall be written and followed for drug product containers and closures.
- 211.100 (a)—There shall be written procedures for production and process controls and they shall be reviewed and approved by the quality control unit.
- 211.110(c)—In-process materials shall be tested for identity, strength, quality and purity as appropriate and approved or rejected by the quality control unit.
- 211.113(a)—Appropriate written procedures shall be established to prevent objectionable organisms in nonsterile drug products.
- 211.113(b)—Appropriate written procedures shall be established to prevent microbiological contamination of sterile drug products.
- 211.160(b)—Laboratory controls shall include the establishment of scientifically sound specifications and test procedures.
- 211.165(b)—There shall be appropriate laboratory testing of each batch of drug product required to be free of objectionable microorganisms.
- 211.165(e)—The accuracy, sensitivity, specificity and reproducibility of test methods shall be established and documented.
- 211.167(a)—There shall be appropriate laboratory testing for each batch of sterile drug product.
- 211.194(a) (2)—The suitability of all test methods shall be verified under actual conditions of use.

In the CGMP amendments proposed by the FDA in 1996, several new definitions (e.g., methods validation, process validation, out-of specification) and new sections were added to update these regulations and clarify the FDA's commitment to the importance of validation in the production of pharmaceutical products.

The FDA has also issued two Inspection Guides that are pertinent to our subject: (1) Guide to Inspections of Pharmaceutical Quality Control Laboratories (FDA 1993a) and (2) Guide to Inspections of Microbiological Pharmaceutical Quality Control Laboratories (FDA 1993b).

These guides contain general instructions to FDA inspectors on conducting product-specific inspection audits to measure compliance with the CGMP requirements and with the manufacturer's applications. These may be New (or Abbreviated) Drug Applications (NDAs or ANDAs) or Biologics License Applications (BLAs), and the inspections may be pre-approval (PAI) or biennial inspections. The FDA inspectors are required to observe the laboratory in operation to evaluate compliance with CGMPs and with the manufacturer's commitments in the above applications. They will examine the standard operating procedures (SOPs) for completeness, and the actual test procedures must conform to the written SOPs. If all laboratory procedures are compendial, then validation data can be minimized, although many manufacturers perform full validation studies as a matter of course. Alternative assays must be fully validated; the data for these studies will be closely examined by the regulators. There is considerable guidance available on the validation of nonmicrobiological (e.g., chemical) test methods that provide specific instruction regarding the demonstration of equivalence between new and existing methods. Some examples include Chapter 1225 of USP 25 (USP 2002a) and ICH documents Q2A and Q2B. In contrast, very little guidance specific to the validation of microbiological testing has been published. The inherent variability of microbiological assays makes them more difficult to validate than other assay procedures. Lack of specific guidelines for proper validation also makes it more difficult for an FDA inspector or an FDA microbiologist, reviewing test documentation, to assess whether an alternative method has been adequately validated. The authors of this book hope that documents such as the PDA Technical Report mentioned earlier and Chapter 12 herein will help to shed light on this problem and make it easier to validate microbiological assays in the future.

Although both Inspection Guides are informative, the Guide to Inspections of Microbiological Pharmaceutical Quality Control Laboratories (FDA 1993b) gives more insight into the information that the FDA will require when inspecting microbiology laboratories. The guide states that, along with other nonsterile products, the FDA has "…seen a number of problems associated with the microbiological contamination of topical drug products, nasal solutions and inhalation products." The guide also quotes Chapter 1111 of USP 25 (Microbiological Attributes of Non-Sterile Pharmaceutical Products: USP 2002b) as follows: "The significance of microorganisms in non-sterile pharmaceutical products should be evaluated in terms of the use of the product, the nature of the product, and the potential hazard to the user." The FDA expects each manufacturer to develop microbiological acceptance criteria for their nonsterile products by using product-specific USP

monographs, USP General Chapters (e.g., <61>, Microbial Limit Tests), and the scientific literature to determine which organisms are objectionable in the product.

For example, gram-negative organisms such as *Burkholderia* (formerly *Pseudomonas) cepacia* are considered objectionable in many products, but there are no test methods in the USP for this organism, so the manufacturers are expected to develop their own assay. The guide also asks inspectors to examine the manufacturer's methods for recovery of damaged organisms by inactivating preservatives in the product or using different incubation times and temperatures. FDA laboratories use the test procedures in the *Bacteriological Analytical Manual* (*BAM* 8th edition; FDA; CFSAN; AOAC International) to "… optimize the recovery of all potential pathogens and to quantitate and speciate all recovered organisms." Manufacturers would do well to imitate this practice so that they can identify any potential problems in their products before release, thus heading off possible regulatory action by the FDA in the future.

Part V of the Guide to Inspections of Microbiological Pharmaceutical Quality Control Laboratories titled Methodology and Validation of Test Procedures, advises the inspectors to "…evaluate the methodology for microbiological testing…" and, if it is a PAI, to "…compare the method being used against the one submitted in the application." It also repeats the USP statement that "…an alternate method may be substituted for compendial tests, provided it has been properly validated as giving equivalent or better results." Other than stating that it "…would be virtually impossible to completely validate test procedures for every organism that may be objectionable..," the FDA gives no specific guidance here on how to evaluate the validation data that a manufacturer might present. The final decision is left to the discretion of the inspector. This has been an item of concern to those manufacturers who feel that FDA inspectors may not always have the technical knowledge needed to understand and evaluate the newer technology.

The guide does reference the use of automated microbial identification systems and states, "The utilization of automated systems for the identification of microorganisms is relatively common in the parenteral manufacturer where isolates from the environment, water systems, validation and people are routinely identified." The FDA is also using these systems in its own laboratories and has considerable experience in validating them.

Finally, Guidance on General Principles of Process Validation (FDA 1987) and Guidance on Sterile Drug Products Produced by Aseptic Processing (FDA 1987) give further insight into the elements and concepts the FDA considers as acceptable parts of a validation program. The former document states, "Although the particular requirements of process validation will vary according to…the nature of the…product (e.g., sterile vs. non-sterile) and the complexity of the process, the broad concepts stated in this document have general applicability and provide an acceptable framework for building a comprehensive approach to process validation."

The most recent legal precedent in the United States that relates to laboratory testing is the now-famous Barr decision (United States v. Barr Laboratories, Inc. 1993). This decision dealt primarily with failing test results (called "out-of-specification" results or OOS by the court), averaging test results, retesting, and validation studies. Many of the findings provide specific guidance on what actions the FDA can require from quality control laboratories within the context of the CGMP regulations. The FDA has also incorporated this analysis of the CGMPs into its Guide to Inspections of Pharmaceutical Quality Control Laboratories and as part of the 1996 proposed amendments to the CGMP regulations. The Barr decision has had a major impact on what was regarded as "standard industry practices" regarding OOS results, retesting, and failure investigations. The latter have become a class of documents that must be maintained by the manufacturer and are subject to review during inspections by regulatory agencies. In the part of the ruling related to assay validations, the court began by reiterating the requirements in the CGMPs for assay validation and repeated the statement that the suitability of testing methods must be verified under actual conditions of use. The court ruling also found that a manufacturer can show that its methods are validated in one of three ways: "(1) If the method was approved as part of its ANDA; (2) if the method is the same as that used in the current version of the USP; or (3) if a firm validates its method through a validation study. If the method falls into one of these three categories, firms need only show that the method works under conditions of actual use. However, if firms either adopt methods the USP does not recognize or modify USP procedures, they must validate these procedures. Systems suitability data alone are insufficient for validation. Statistics in itself is absolutely not a validation process. Statistics merely aids in the evaluation of the validation data." If we wanted to further generalize the spirit of this ruling, we would substitute other registration documents for the term "ANDA" and other compendia for the USP.

Subsequent to the court ruling, the two ICH Guidelines mentioned previously (Q2A and Q2B) were published in the *Federal Register;* they describe the validation of analytical procedures. Thus, manufacturers who choose to use the new rapid microbiological methods as alternate test methods must realize that they will be subject to the same (or greater) regulatory scrutiny regarding OOS results, retesting, averaging, and failure investigation as they would be if they were using classical methods.

Laboratory control issues such as environmental monitoring, raw material, and component testing and sampling procedures have been a constant source of inspection citations from the FDA (known as "483 items" from the FDA form on which they are written) and other regulatory authorities. Recent presentations by personnel from the Medicines Control Agency (MCA) in the United Kingdom and the FDA show that these issues are still at or near the top of CGMP deficiencies found at both U.S.

and European pharmaceutical manufacturers. Both CBER and FDA field inspectors listed the following as two of their most frequent citations during presentations at PDA meetings in 1997 and 1998: "Failure to properly validate microbial test methods" and "Laboratory controls not based on scientifically sound and appropriate specifications." Because the majority of the rapid microbiological methods are intended for use in these types of assays (e.g., water testing, environmental monitoring, microbial limit testing, and antimicrobial effectiveness testing), it stands to reason that they will continue to be a focus of CGMP inspections.

REGULATORY ACCEPTANCE

In the General Notices section of USP 23, under the heading Tests and Assays—Procedures is the statement that alternative test methods may be used to determine compliance with USP standards. These alternative methods may be "…chosen for advantages in accuracy, sensitivity, precision, selectivity or adaptability to automation or computerized data reduction or in other special circumstances. Such alternative…procedures or methods shall be validated. However, …where a difference appears or in the event of a dispute, only the result obtained by the procedure given in this Pharmacopeia is conclusive." A similar statement appears in the European Pharmacopoeia General Notices Assays and Tests: "The assays and tests described are the official methods upon which the standards of the Pharmacopoeia depend. The analyst is not precluded from employing alternative methods in any assay or test if it is known that the method used will give a result of equivalent accuracy. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative." The Japanese Pharmacopoeia General Notices also state, "The test methods of the Japanese Pharmacopoeia can be replaced by alternative methods which give better accuracy and precision. However, where a difference is suspected, only the results obtained by the procedure given in this Pharmacopoeia are effective for the final judgment."

All of the major pharmacopeias worldwide operate on a "continuous revision process," in which changes to monographs or general information chapters are proposed by their expert committees, reviewed, published for comment, and then published as revised or supplemental documents. The USP may publish several supplements during the actual publication cycle of the Pharmacopeia itself. The frequent publication of *Pharmacopeial Forum* (six times per year) allows the USP to rapidly move new ideas or proposed changes to chapters into the public view for comment and subsequent acceptance (or rejection) as an official change.

Regulatory concerns and pressures are often the principle reasons why drug manufacturers do not change to more cost-effective or technologically advanced systems in the pharmaceutical industry. CBER and CDER of the FDA do not approve test methods, per se, but do approve applications for new products (NDA, ANDA, BLA) or supplements to the file for existing products. When the holder of a product application wishes to change specified microbiology tests for a product or component, it is often necessary to submit to the file a description of the test and acceptance limits for the product, as well as information demonstrating the appropriateness of the test.

FDA regulations state that equivalent methods are acceptable (e.g., 21 CFR 610.9) but the agency felt that further clarification was needed for manufacturers to report changes in production or testing methods to the FDA. The agency published a Final Rule, Changes to an Approved Application, in which it amended two existing regulations (21 CFR 601.12 and 314.70) dealing with changes to be reported for biological and pharmaceutical products, respectively (FDA 1997b). The rule identifies three categories of change, each requiring a specific level of reporting depending on the importance (major, moderate, or minor) of the change. These categories are as follows.

Category 1—Major changes that have a substantial potential to have an adverse effect on the identity, strength, quality, purity, or potency of the product. These changes require a supplement submission to the FDA and approval prior to distribution of the product affected by the change (Prior Approval Supplement).

Category 2—Moderate changes that have a moderate potential to have an adverse effect on the identity, strength, quality, purity, or potency of the product. These changes require a supplement submission to the FDA at least 30 days prior to product distribution (Changes Being Effected Supplement), unless this time period is specifically waived.

Category 3—Minor changes that have only a minimal potential to have an adverse effect of the identity, strength, quality, purity, or potency of the product. These changes may be described in an Annual Report.

Examples of changes that fall into each of these categories can be found in two FDA Guidance Documents, Changes to an Approved Application for Specified Biotechnology and Specified Synthetic Biological Products (FDA 1997c) and Changes to an Approved Application: Biological Products (FDA 1997d). A substantial number of examples are listed in these documents, but the one that seems most pertinent to our discussion of regulatory acceptance of rapid microbiological methods is listed in both documents under Part II, "Changes under 21 CFR 601.12(b) and 314.70(g)(1), Changes requiring supplement submission and approval prior to distribution of the product made using the change (major change): Any change in manufacturing processes or analytical methods that…establishes a new analytical method…." Thus it seems that the current thinking at the FDA is that a change establishing a new analytical method has a significant potential to have an adverse effect on the identity, strength, quality, purity, or potency of a product and needs to be approved by the agency before use. However, some may

argue that another example that could apply equally well is listed under Part IV, "Changes under 21 601.12(d) and CFR 314 (g)(3)–Changes to be described in an annual report: Establishment of an alternate test method for reference standards, release panels or product intermediates, except for release testing of intermediates licensed for further manufacture." The question then becomes whether a rapid microbiological method is a "new regulatory analytical method" or an "alternate analytical method" for the particular process for which it is proposed by a manufacturer. If the manufacturer intends to replace the "classical" method with the rapid method, it may be difficult to represent this to the FDA as an alternate method. Obviously there is some ambiguity here, but the prevailing wisdom should tell the prudent manufacturer to submit the method as a Category 1 Supplement and obtain approval before effecting a permanent change.

The two Guidance Documents also describe a vehicle that can be used to make the reporting process easier. This is the Comparability Protocol described in 21 CFR 314.70(g)(4) and 601.12(e). This document is a supplement that "establishes the tests to be done and the acceptable limits to be achieved to demonstrate the lack of adverse effect for specified types of manufacturing changes on the safety and effectiveness of a product." A comparability protocol itself requires FDA approval prior to its implementation, because it may result in decreased reporting requirements for all changes that it covers. In general, the reporting requirement will decrease by at least one category. Thus, if a manufacturer received approval for a comparability protocol to validate new microbiological methods, any test method following that protocol could probably be submitted under Category 2.

Those who feel that the process of using a comparability protocol is too cumbersome should submit the validation data from their new method(s) to the FDA as a change to their application (Category 1). If the new method has been described in a scientific journal article, these data could be used to support the application. The manufacturer must clearly demonstrate that the new method is as good as the standard or current method by comparing the two in the conventional style of a scientific study, including defined acceptance criteria and the use of proper experimental controls to demonstrate the accuracy, precision, and specificity of the test. Ultimately, the new microbiological method must be suitable for assessing the specific product attribute.

A Proposed Rule (FDA 1999a) and Guidance Document (FDA 1999b) further amend the above regulations by adding a fourth reporting category. This is a moderate change for which product distribution can begin when the FDA receives the supplement (i.e., no 30-day waiting period). The Guidance Document also expands the list of manufacturing changes that are affected by the regulation. This regulation has not been finalized as of this writing, so the reader should check the current status of the regulation to get the latest information from the FDA regarding manufacturing changes.

The situation in the European Union (EU) with respect to approval of changes to marketing authorizations (or variations, as they are called by the EU) is similar to that described above for the FDA but has some important differences. In 1995, the EU adopted a new system for variations (European Commission [EC] 1995a, 1995b). The objective, as stated in the EC Regulations is "to provide speedy and efficient procedures for approval of changes to marketing authorizations, whilst maintaining adequate safeguards to public health." Under this new system, all former categories of variations have been replaced by two new categories: Type I and Type II.

The definition of a Type I variation is given as a list of 33 "minor changes"; each requires that specific conditions be met. Some examples that are pertinent to our discussion concerning rapid microbiological methods are as follows.

- Change in the test procedures of the active substance. Condition: Results of method validation show new test procedure to be at least equivalent to the former procedure.
- Change in the test procedures of the medicinal product. Conditions: Medicinal product specifications are not adversely affected; results of method validation show new test procedures to be at least equivalent to the former procedure.
- Changes to comply with supplements to pharmacopeias. Conditions: Change is made exclusively to implement the new provisions of the supplement.

The manufacturer must submit supporting documentation for these Type I variations following the general principles set out in the EC Regulations. These state that "Type I variations are by definition minor changes but accompanying data and/or commitments concerning ongoing data and relevant assurances will be necessary in many cases." Some points suggested as relevant to the documentation are the following.

- Adequate justification for the change, including development pharmaceutics data where appropriate.
- Assurance that the methods of analysis and specifications being used are as currently authorized.
- Analytical validation data for all new methods and comparative analytical data on the existing method.

Type II variations are defined (by exclusion) as "all other changes, which are neither covered by the Type I list nor by the definition of changes which require new marketing authorization." Changes in this category are broadly equivalent to Class 1 changes as defined by the FDA.

Thus we can see that the change to rapid microbiological methods would be considered as a Type I variation in the EU. The regulatory authorities in the EU have committed to processing these variations within 30 days of receipt of a valid application. This would be significantly faster than the processing time for a Category 1 submission to the FDA, normally six months. If the FDA could be persuaded to consider changes in this type of analytical method as a Category 2 submission, both regulatory authorities would be on the same review timeline.

SUMMARY AND CONCLUSIONS

We have looked briefly at the issues and concerns of regulatory authorities (with special emphasis on the FDA) in dealing with changes from standard to rapid microbiological test methods in the pharmaceutical industry. Both the U.S. and EU CGMP regulations place a strong emphasis on validation of manufacturing processes and assay methods. The Barr decision in the United States has given additional emphasis to this issue as well as consideration of retesting and OOS results. CGMP inspections will continue to focus strongly in these areas.

While current documents from the FDA, EU, and ICH give general guidance regarding validation and reporting requirements, it will take some time before more specific guidelines are accepted for microbiological test methods. Until then, manufacturers must continue to rely on sound scientific principles to generate adequate validation data. They should also communicate regularly with the appropriate regulatory authority to receive the latest guidance on these issues.

With the recent publication of regulations in the United States and EU concerning the reporting of changes (variations) to a product application (authorization), this process has become clearer and, to some extent, easier.

Many of the same challenges discussed in this chapter were encountered and overcome during the development, licensing, and acceptance of the *Limulus* amebocyte lysate (LAL) assay for endotoxin as a replacement for the rabbit pyrogen test (see Chapter 11). In particular, Hochstein and Novitsky describe the cooperative efforts of the FDA, USP, WHO, and the pharmaceutical industry to produce data and documentation that facilitated acceptance of this "new technology." Much of the groundwork for validation and acceptance of rapid microbiological methods has been started. I believe that, in a relatively short time, the use of rapid microbiological methods will be routine in the pharmaceutical industry and accepted by regulatory authorities worldwide.

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14
New Technologies for Microbiological Assays

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Rapid and specific detection of a single organism or a very low number of microorganisms is a continuing challenge in the bioanalytical sciences. Refining the detection to be selective for viable organisms introduces a further level of difficulty into an already arduous analytical problem. Medical microbiology, food testing, pharmaceutical quality control, and biological warfare detection all share a need for methods that would detect one or a few microorganisms. In medical microbiology, rapid diagnosis of infection is important for the timely administration of appropriate therapeutic measures and isolation of infectious patients. Safety of the food supply requires real-time monitoring of raw and processed food materials. Likewise, microbiological contamination of pharmaceutical preparations must be accurately assessed during production and storage. Finally, the threat of biological warfare and terrorist attacks involving biological warfare agents requires rapid and accurate identification of potentially harmful agents in the battlefield or in a domestic environment.

Traditional culture methods for detecting microorganisms are slow and time consuming. Alternative, nongrowth analytical strategies for detection of microorganisms include direct detection of surface antigens, cell wall components (e.g., calcium dipicolinate) (Pellegrino et al. 1998), and intracellular components such as nucleic acid sequences (Matthews 1993), enzymes (e.g., beta-glucuronidase, adenylate kinase) (Chang and Huang 1997; Corbitt et al. 2000; Squirrel and Murphy 1994), and cofactors (e.g., ATP [adenosine triphosphate]) (Stanley et al. 1997).

These methods can be enhanced by preanalytical isolation and concentration using magnetic beads coated with antibodies specific for a microorganism (Taylor et al. 1997; Widjojoatmodjo et al. 1991; Wolfhagen et al. 1994). This reduces interference from components of the sample matrix and contributes to increased sensitivity and specificity.

The current range of microbiological assays cannot meet the analytical goals that are now being formulated for the most ambitious microbiological tests of the future—i.e., rapid detection, rapid identification, differentiation of viable from nonviable organisms, and low-cost detection using a small portable analyzer.

This chapter explores a series of technological developments that may provide a route to improved microbiological tests through new detection methods or assay formats (see Figure 14.1).

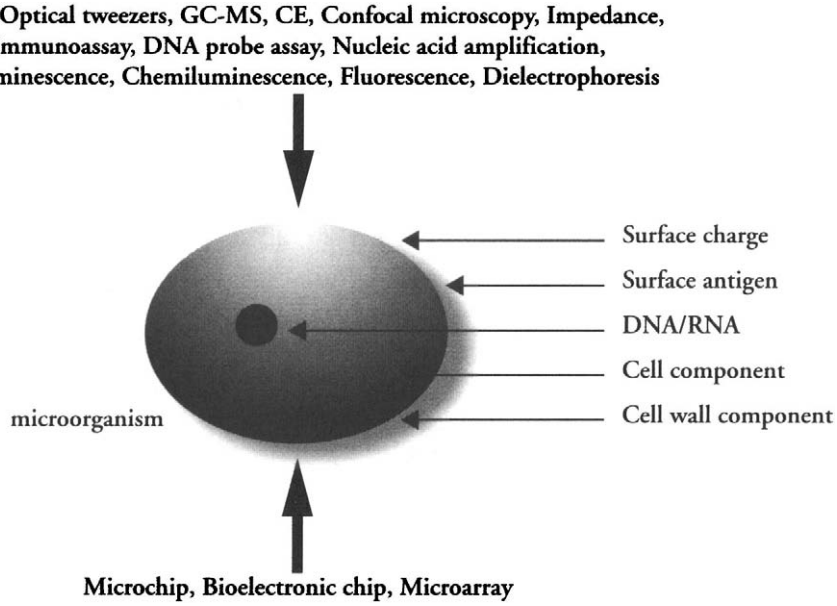
IMMUNOLOGICAL TESTS

Molecular recognition by an antibody molecule provides the basis of sensitive, specific, and versatile assay methods (Kricka 1994). Sandwich-type immunoassays are used to detect microbial antigens and thus confirm the presence of the microorganism. Most methods are relatively slow and have limited sensitivity, but recent developments in immunoassay technology offer improved sensitivity and convenience through signal amplification, background reduction, or a combination of these factors. These have not yet been fully explored in the context of microbiological testing. A further line of development may be to take advantage of oligonucleotides that have binding properties for ligands. These are produced through combinatorial chemistry and systematic evolution and enrichment processes. Such reagents have been tested in an enzyme-linked immunoassay and shown to be effective; they may offer benefits as alternatives to the conventional antibody type of molecular recognition molecules (Drolet et al. 1996).

Signal Amplification

Selection of labels with higher specific activity or more sensitive label detection can improve sensitivity in sandwich-type immunoassays (Pringle 1993).

Figure 14.1 Analytical strategies for microbiological assays



AFM is atomic force microscopy; CE is capillary electrophoresis; GC-MS is gas chromatography-mass spectrometry

Bioluminescent and chemiluminescent labels and detection schemes

Acetate kinase is just one example of a new label that can provide increased sensitivity in sandwich assay formats (Murakami et al. 1994). This label is detected in a coupled bioluminescent reaction in which the acetate kinase acts on acetyl phosphate and ADP (adenosine triphosphate) to produce ATP, and then the ATP is detected using a mixture of firefly luciferase-firefly luciferin—Mg^{++}. The detection limit for the acetate kinase label is 8.6 zeptomoles (8.6×10^{-21} moles), which corresponds to less than 6000 molecules of the enzyme.

Luciferases and photoproteins are attractive as immunoassay labels because of the sensitivity of bioluminescent detection reactions for these proteins. A further advantage is that the genes for many bioluminescent proteins have been cloned. These genes can be spliced with the genes for other molecular recognition proteins (e.g., protein A, IgG heavy chain). This provides a route to a reproducible supply of active fusion conjugates that retain the biological activity of the bioluminescent protein and the molecular recognition properties of the protein (Frank et al. 1996; Kobatake et al. 1993; Lindbladh et al. 1991; Zenno and Inouye 1990). An exciting prospect is to use this gene fusion technique to produce multiply labeled binding proteins—e.g., $(\text{luciferase})_n$-antibody. This type of fusion conjugate has the potential for very high specific activity that could translate into improved assay sensitivity. The following are some recent examples of fusion proteins, including luciferase and photoprotein fusions, developed for analytical purposes.

- apoaequorin—IgG heavy chain
- apoaequorin—Protein A
- bacterial alkaline phosphatase—IgG Fc binding protein
- bacterial alkaline phosphatase—synthetic octapeptide
- bacterial alkaline phosphatase—anti-HIV 1 gp 41 scv (single-chain antibody)
- bacterial alkaline phosphatase—human proinsulin
- beta-galactosidase—interferon-alpha 2
- beta-galactosidase—B19-specific oligopeptide
- core-streptavidin—single-chain antibody (scFv)
- firefly luciferase—RNA binding protein
- firefly luciferase—Protein A
- human placental alkaline phosphatase–4-1BB ligand
- marine bacterial luciferase (beta-subunit)—protein A
- metapyrocatechase—protein A
- obelin—proZZ
- *Pyrophorus plagiophthalamus* luciferase—protein A

Another route to improved immunoassay sensitivity is to increase the sensitivity of the detection reaction for a label. Chemiluminescent detection methods for horseradish peroxidase and alkaline phosphatase labels have provided dramatic increases in sensitivity over the conventional colorimetric assay procedures: as little as 1 zeptomole (10^{-21} moles, 602 molecules) of alkaline phosphatase can be detected by a chemiluminescent assay procedure based on an adamantyl 1, 2-dioxetane substrate (Edwards et al. 1994; Kricka 1991; Thorpe and Kricka 1986). The chemiluminescent substrates used for enzyme detection have a low chemiluminescence quantum yield (<1%). This limitation provides an avenue for further improvement in sensitivity, and work is being directed to the synthesis of chemiluminescent detection reagents with improved light-emission characteristics and higher quantum yields—e.g., 7-phenyl-8-hydroxypyrido [3, 4d] pyridazine-l, 4(2H,3H) dione (Ii et al. 1993).

Immuno-PCR and DNA labels

This is one of the newer immunoassay labels, and its appeal is the availability of the exquisitely sensitive polymerase chain reaction (PCR) for label detection (Case et al. 1997; Joerger et al. 1995; Kakizaki et al. 1996; Maia et al. 1995; Numata and Matsumoto 1997; Sano et al. 1992). Double-strand DNA (size range 261 bp–2.67 kb) and single-strand DNA can be used as the label, and after completion of the immunological steps in the immuno-PCR assay, the DNA label is amplified in a PCR reaction. Each bound DNA label is amplified by many million-fold in a typical 25 cycle procedure. Detection limits of less than 600 molecules are possible (see Table 14.1) and the immuno-PCR strategy produces a 104–105 increase in sensitivity compared to a conventional ELISA (enzyme-linked immunosorbent assay) (Sperl et al. 1995). Immuno-PCR has been applied to the detection of *Helicobacter pylori* in feces (sensitivity 80.9%, specificity 100%) (Monteiro et al. 2001), and to diagnose influenza (Ozaki et al. 2001).

Background

Another way of improving detection limits is by reducing assay background. Several background-reduction strategies have been developed. Fluorescent lanthanide chelates have a long-lived fluorescence emission that can be distinguished from shorter-lived background emission by

Table 14.1 Immuno-PCR Reactions

Analyte	Detection limit
Anti-apolipoprotein E	1,881 molecules (0.5 fg)
Beta-galactosidase	6,022,520 molecules (10 amol)
Beta-glucuronidase	0.5 ag
Bovine serum albumin	580 molecules
Hepatitis B surface antigen	0.5 pg
Human chorionic gonadotropin	6,022,520 molecules (10 amol)
Human proto-oncogene ETSI	5,780 molecules
Pasturella piscicida	3.4 CFU/mL
Thyrotropin	602,252 molecules (0.1 amol)
Tumor necrosis factor alpha	21,687,456 molecules (0.625 pg)

introducing a delay between excitation and signal measurement. A recent microparticle-based time-resolved fluoroimmunoassay for prostate specific antigen (PSA) using 50 µm diameter latex microparticles illustrates the sensitivity of this method (Lovgren et al. 1997). As few as 11,000 molecules of the europium chelate label could be detected on an individual particle, and in the immunoassay, the detection limit for PSA was 0.05 attomoles. The instrumentation provided illumination to only 10% of the particle surface, so there is room for further signal acquisition and hence improved sensitivity with this method.

A new variation on the quantitation of radioisotopic labels in immunoassay measures the two-photon emission from a ^{125}I (iodine) label. Restricting detection to only two photon events dramatically reduces the background count to 0.5 counts per week, compared to the 20–70 counts per minute with traditional counting methods. The detection limit for ^{125}I using this multiphoton detection strategy is 0.1 zeptomoles (60 molecules). Results obtained in sandwich immunoassays (SIRMAs, supersensitive immunoradiometric assays) using this new counting strategy illustrate the sensitivity of the method. A SIRMA assay for thyrotropin was 50,000 times more sensitive than a standard IRMA (1 fg/mL versus 55 pg/mL) (Drukier 1998).

Another approach to improving immunoassay sensitivity is the immune complex transfer strategy. This utilizes two assay tubes and a doubly labeled capture antibody (dinitrophenol [DNP], biotin). Sample and conjugate incubation steps of the sandwich immunoassay are performed in an anti-DNP antibody coated tube to which the labeled capture antibody is bound via the DNP label. After completion of the incubations with sample and conjugate, DNP-lysine is added to dissociate the immune complex. This is transferred to a second streptavidincoated tube, and the immune complex is captured via the biotin label on the capture antibody. The net effect is that any non-specifically bound conjugate is left behind in the first tube and does not contribute to the measured signal in the second tube. Immune complex immunoassays for ferritin detected as little as 1 zeptomole (602 molecules), but this sensitivity is achieved at the cost of increased assay time and experimental complexity (Hashida and Ishikawa 1990; Ishikawa et al. 1993).

Formats

Immunoassays have been developed in a range of formats for use in laboratories and for use in extra-laboratory settings (e.g., doctors office, bedside, clinic, streamside). Simplification of immunoassays is important for the latter application, and various hand-held disposable immunoassays for microorganisms are now available (e.g., Biosite Diagnostics Triage^{R} *Clostridium difficile* test panel).

Capture and enrichment

A recent format is the enzyme capture assay (ECA). This utilizes an immobilized antibody against a cellular enzyme to capture enzyme molecules released from an organism (e.g., betaglucuronidase, beta-galactosidase). The antibody-bound enzyme is then assayed using an appropriate substrate (Huang et al. 1997); e.g., *E. coli* can be detected by using an immobilized anti-beta-lucuronidase and a fluorigenic beta-glucuronidase substrate. Other capture strategies use small cross-flow hollow fiber membrane-based sampling devices (GLOWGRUB™, FSM Technologies, United Kingdom). These have been designed to capture *E. coli* 0157 and *Staphylococcus aureus,* and the unit is compatible with either a colorimetric or luminescent detection technology.

Biosensors

Immunological reagents can also be combined with different sensing technologies to produce immunosensors. For example, enzyme-linked amperometric immunosensors have been developed for the detection of *Salmonella* (Brooks et al. 1992), and a piezoimmunosensor has been developed for the detection of *Salmonella typhimurium* (Luong et al. 1990; Prusak-Sochaczewski et al. 1990).

Adapting immunoassays for monitoring microorganisms in the air is a prominent military objective. An immunobiosensor mounted in a remotely piloted aircraft has been designed and flight-tested for airborne detection of aerosolized biological warfare agents. This sensor is based on a fiber optic probe onto which antibodies specific for biological warfare agent simulants are immobilized. The simulant binds to the immobilized antibodies, and bound simulant in turn reacts with a fluorescently labeled detection antibody. Interrogation of the fiber optic probe for bound fluorophore provides an indication of the presence of the reagent (Anderson et al. 1997). Assays for microorganisms have also been accomplished by using a wave guide coated with specific antibodies. The sample is stained with a fluorescent dye (e.g., Nile red, ethidium iodide, acridine orange), and then the wave guide is dipped into the sample; bound stained microorganisms are detected by fluorescence measurements. Assays for *Bacillus anthracis* and *Salmonella* detected 3 cells/µL (approximately 1,000 cells) (Ligler et al. 1991, 1996).

Homogeneous assays

Any immunoassay strategy that leads to a homogeneous (nonseparation) format is highly desirable because it eliminates the serial washing and reagent addition steps in the usual separation type of immunoassay. Most homogeneous immunoassays have limited sensitivity, so a sandwich assay that combines a homogeneous format and ultrasensitivity represents an important breakthrough. The luminescent oxygen channeling immunoassay (LOCI) uses two populations of microparticles (250 nm diameter) each coated with one of a matched pair of monoclonal anti-bodies (Ullman et al. 1994). One population of particles is impregnated with a dye (bromosquaraine) and the other with a mixture of a chemiluminogenic molecule (thioxene) and a fluorophore (europium chelate). A test antigen reacts with the particle-bound antibodies to form a bridge between two different particles. Laser irradiation of the reaction mixture produces singlet oxygen at the surface of the dye-loaded particle, and this diffuses to the adjacent particle, where it reacts to produce a dioxetane that decomposes and transfers energy to the fluorophore, which then emits light. This process can occur only if the test substance binds the two particles together. Any singlet oxygen generated in solution is quickly deactivated and thus cannot contribute to the light emission

signal. Rapid nonseparation assays for various molecules have been developed (e.g., 12 minute assay for thyroid-stimulating hormone [TSH], detection limit of <0.0125 mlU/L); this assay design has considerable potential for detecting microorganisms through surface antigens.

CELL COMPONENTS

Measurement of intracellular ATP or adenylate kinase provides a general method for microorganism detection and enumeration. The bioluminescent ATP method detects approximately 1,000 cells (a bacterial cell typically contains 1 fg of ATP). This detection technology can be used effectively with a compartmentalized membrane through which the sample is filtered (Millipore, MicroStar™, and MicroCount Digital™). Microorganisms distributed in the >600 compartments in the membrane are lysed, and released ATP is detected by using the firefly luciferase-luciferin reaction. Light emission is detected and quantitated using a charge-coupled device (CCD) (detection limit of 1 CFU). Modifications of the basic firefly luciferase-luciferin technology, using a myokinase and pyruvate kinase coupled cycling reaction, lowers the detection limit to 100 cells (Hawronskyj et al. 1994).

Lower detection limits are possible with the adenylate kinase method. Adenylate kinase released from cells (600 molecules of adenylate kinase per bacterium, 0.1% of cell protein) is reacted with ADP to form ATP, and this is measured using the bioluminescent firefly luciferase reaction (Squirrel and Murphy 1994). The assay has a detection limit of 34 cells, and the assay can be modified for small reaction volumes (5 μL) on microscope slides or in wells. Extending the incubation period for ATP generation (60 minutes) reduces the detection limit to 4 cells on a slide and 1 to 2 cells in a microwell obtained (Squirrel and Murphy 1994).

Enumeration of microorganisms via cellular hydrolase enzymes (e.g., alkaline phosphatase, beta-galactosidase, glucuronidase) is usually accomplished with chromogenic or fluorigenic substrates. Chemiluminescence offers an alternative and more sensitive detection option. The phenylgalactose-substituted chloroadamantyl 1, 2-dioxetane and chloroadamantyl 1, 2-dioxetane substrates (Tropix Inc., United States) for beta-galactosidase detect 2–8 fg of this enzyme, as compared to a detection limit of 2 pg with the methylumbelliferyl-beta-D-galactopyranoside fluorescent substrate (Bronstein et al. 1997; Jain and Magrath 1991). In one example of the use of this type of substrate to measure cellular beta-galactosidase, a single coliform was detected in a 100 mL sample after 6–9 hours of incubation, permeabilization of cells in the sample by using polymyxin B, and a 45 minute chemiluminescent assay (Van Poucke and Nelis 1995). Alternative chemiluminescent enzyme substrates include indoxyl and thioindoxyl derivatives, but studies using these substrates in the context of cell detection and enumeration are still at an early stage (Mahant 1996).

Some newer microbiological assay methods target other cellular components (Pellegrino et al. 1998). For example, dormant bacterial forms (endospores) can be detected because of the presence of calcium dipicolinate in cell walls (Pellegrino et al. 1998). Addition of terbium chloride leads to the formation of a highly fluorescent terbium dipicolinate complex detected by fluorometry. This method was applied to *Bacillus globigii* endospores and detected 1.12×10^5 CFU/mL. There is scope for further elaboration of this type of method by targeting other cellular components that can form fluorescent products with lanthanide ions.

MICROCHIPS

Various microminiaturization technologies (photolithography, hot embossing, reactive ion etching, microinjection molding) are being explored for the production of microanalyzers (van den Berg and Bergveld 1995; Kricka and Wilding 1996; Kricka et al. 1994). These “lab-on-a-chip” devices, designed to test microvolumes of a sample, are typically fabricated on 1 cm×1 cm silicon, glass, or plastic chips. Microminiature versions of several conventional analyzers include PCR microreactors, liquid chromatographs, capillary electrophoresis analyzers, and flow cytometers (Colyer et al. 1997; van den Berg and Bergveld 1995). Advantages of microanalyzers include low consumption of sample and reagent (microliters to nanoliters) and integration of all the steps in a complex analytical procedure into a single device. For example, white-cell isolation and PCR analysis can be successfully integrated in a single microchip for DNA analysis (Wilding et al. 1998).

Cell Analysis Chips

Micromachined and micropatterned structures provide unique opportunities for manipulating and analyzing biological cells. For example, red-cell deformability can be measured in silicon microchannels (Tracey et al. 1995), and cell filtration and selection can be achieved by using silicon microfilters constructed from silicon posts, weirs, and microchannels (Wilding et al. 1998). So far, the potential of microchip-based analyzers for microbiological assays has not been explored extensively. Modulation of fluid flow in silicon microchannels as a result of microbial growth has been explored as one means of detecting the presence of microorganisms in a sample (Wilding et al. 1997).

Cell manipulation and selection can be achieved by using different types of electrode systems in microfabricated chambers. *E. coli* can be moved around a network of capillary channels (15 μm×55 μm cross section) at speeds of up to 0.5 mm/sec by electro-osmotic and/or electrokinetic pumping (Li and Harrison 1997).

A bioelectronic chip containing an array of 25 platinum electrodes (80 μm diameter, 200 μm spacing) provides a means for dielectrophoretic isolation of *E. coli* from whole blood (Cheng et al. 1998). Nucleic acid is released from captured cells by applying a 500 volt pulse to the electrodes, followed by proteolytic digestion with proteinase K. Other microorganisms, such as *Micrococcus lysodeikticus* and *Staphylococcus epidermis,* have also been isolated from complex media by using this type of biochip. This type of chip has considerable potential for sample preparation and for hybridization analysis of the released nucleic acid.

Dielectric methods have also been employed to separate cells, enrich for selected cell types, assay for microbial contamination, and differentiate viable from nonviable cells (Fiedler et al. 1998; Markx et al. 1996; Pethig and Markx 1997; Stephens et al. 1996; Talary et al. 1995; Zhou et al. 1995). For example, recent work exploits opposing arrays of platinum-titanium and indium tin oxide microelectrodes to manipulate cells in 30-μm-deep glass microchannels. Forces generated by an alternating current field under conditions of negative dielectrophoresis direct cells away from the electrodes to regions of low field strength. Results with mammalian cells are encouraging, and these devices may also have potential to manipulate microorganisms, and therefore produce microscale assays (Fiedler et al. 1998). Electrode arrays have also been used to assess the viability of *E. coli* attached to 6-μm-diameter latex beads. Viability was assessed from electrorotation measurements in an electrical field modulated from 100 Hz to 5 Mhz. This type of technology can be used to assay the concentration and viability of water-borne microorganisms. The dielectric properties of a latex bead reagent coated with a specific binding agent are modulated when it is complexed to the target analyte; this can be monitored by its electrorotation response (Burt et al. 1996).

Microarrays

Microfabrication technologies (ink-jet printing, light-directed combinatorial chemistry, microdispensing) are being employed to manufacture arrays of reagents (oligonucleotides, proteins) for massively parallel simultaneous testing (Kodadek 2001; see also http://www.gene-chips.com). One example of this technology is the microspot assay (Ekins and Chu 1997). Antibody dots, typically 10–100 μm, are deposited onto the bottom surface of a plastic well and serve as the capture antibodies in a microimmunoassay. This type of assay differs from conventional sandwich immunoassays in that the amount of capture antibody is very low. The antibody microdot acts like a thermometer and senses the antigen concentration in the sample without markedly altering its concentration. Specific advantages of this new analytical strategy are that the assay is sample volume independent and has excellent sensitivity. An assay for TSH had a detection limit of 0.0002 IU/L (Ekins and Chu 1993). This type of device could be adapted for microorganism detection by using arrays of antibodies specific for microbial antigens.

Sample capacity is important for all of the different types of microanalytical devices. Detection of a few microorganisms in several liters of test fluid would require processing of a large sample volume to ensure analysis of a representative sample. The time taken to pump sample material through devices with capacities in the low microliter range may be restrictive; preanalytical concentration by using, for example, magnetic antibody reagents may be required.

INSTRUMENTAL TECHNIQUES

Various instrument-based methods have been developed for microbiological assays, such as measurements of impedance (Silley and Forsythe 1996), pH (Williams et al. 1990), and oxygen consumption (Stitt et al. 1996). In the latter method, the presence of microorganisms is detected by changes in the concentration of dissolved oxygen as a result of metabolic activity, and oxygen is determined from the quenching of fluorescence emission by oxygen of indicator dyes such as tris-2,2'-bipyridyl ruthenium (II) salts, diphenylanthracene, and tris-4,7-diphenyl-1,10-phenanthroline ruthenium (II) salts. The assay is performed in an indicator microplate prepared by coating the wells of the plate with silicone rubber impregnated with the fluorophore. Samples are then incubated in the coated microwells. Experiments with broths of *E. coli* determined detection limits of 10 CFU/mL after a 7 hour incubation period. A similar electrochemical strategy involves electrochemical detection of reduced reaction products formed by reaction of the contents of cells and a suitable redox indicator (Pasero et al. 1994; Rohrback 1993). Cells are first isolated on a 0.45 μm cellulose acetate filter and then reacted for a methylene blue redox indicator. Reduced methylene blue is then quantitated in an electrochemical measuring cell, and detection limits of less than 1, 000 cells are attained.

Gas Chromatography-Mass Spectrometry

Gas chromatography (GC) can be used to detect and/or identify cells on the basis of the fingerprint of cellular compounds—e.g., cellular fatty acids (Larsson 1994). It can also be used in conjunction with mass spectrometry (MS) to detect organism-unique compounds and to determine "chemical signatures" of microorganisms. This method is used for military applications, to detect microorganisms non-specifically in sampled air (Bruker-Franzen Analytik GMBH, Germany). An air sample is filtered through a quartz-glass filter and then flash pyrolyzed. Pyrolysis products are transferred into a mass spectrometer for analysis. The trend toward smaller and less expensive mass spectrometers (e.g., miniature micromachined mass spectrometers) (Feustel et al. 1995) will no doubt stimulate interest in this type of detection technique.

Atomic Force Microscopy and Optical Tweezers

Direct sensing and manipulation of individual atoms and molecules is now possible because of advances in atomic force microscopy (AFM), atomic scanning microscopy, optical (laser) tweezers, and related techniques. These techniques also have potential in the development of microbiological assays.

In AFM a surface of interest is moved past a microtip (10 nm diameter, microfabricated from silicon or silicon nitride) attached to a flexible cantilever. Deflection of the cantilever as the tip rises and falls in response to the topological features on the surface is detected by using a laser beam. By coating the tip with specific molecules, direct molecular recognition of substances on a surface can be achieved. For example, albumin can be detected on a surface by using an AFM tip coated with anti-human serum albumin (Hinterdorfer et al. 1996). Specific chemical functional groups can also be detected on a surface by using an appropriately sensitized AFM tip ("chemical force microscopy") (Frisbie et al. 1994). AFM and its variants have also proved useful in the study of cells and in surface mapping (Firtel and Beveridge 1995). Some recent applications of AFM include imaging of chromosomes (Jondle et al. 1995), red blood cells (Zachee et al. 1994), DNA molecules (Hansma et al. 1996), and sperm cells (Allen et al. 1995) and estimation of the binding forces between proteoglycans (Dammer et al. 1995).

Optical tweezers (optical trapping) utilize radiation pressure from light incident on an object to trap and manipulate the object (Grimbergen et al. 1993; Uchida et al. 1995). A neodymium: YAG laser is used as the light source (infrared, power up to 150 mW) and can be used with cells without causing damage. The potential of optical tweezers in analysis has been demonstrated in a competitive immunoassay for bovine serum albumin (BSA), in which BSA is covalently coupled to 4.5-μm-diameter latex beads and a glass cover slip coated with mouse monoclonal anti-BSA antibodies as reagents. BSA in a sample competes with BSA immobilized on the beads for anti-BSA antibodies on the surface of the cover slip. The power needed to free a bead from the surface by using the laser tweezers was found to be directly related to the concentration of BSA on the bead (detection limit 1.45×10^{-12} mol/L; concentration range 1.45×10^{-12} to1.45×10^{-15} mol/L). Other applications of optical tweezers include in vitro motility assay (Miyata et al. 1994; Svoboda and Block 1994), single cell and virus isolation (Ashkin and Dziedzic 1987; Grimberger et al. 1993), study of tethered DNA molecules (Perkins et al. 1995), microtubule motors (Wang et al. 1995), movement of receptors in membranes (Sako and Kusumi 1995), and the mechanical properties of cells and cell structures (Bronkhorst et al. 1995).

Single Molecule Assays

Various techniques have been developed to detect and image single fluorophores. These include total internal reflection microscopy, confocal microscopy, fluorescent measurements in levitated microdroplets and hydrodynamically focused solutions, and capillary electrophoresis (Basche et al. 1997; see also http://www.ppc.ethz.ch/sms/). For example, single molecules of B-phycoerythrin can be detected by capillary electrophoresis with a helium-neon laser as the excitation source (Dovichi and Chen 1997). The single-molecule detection sensitivity of these techniques has potential in all areas of analysis, and future adaptation to microbiological assays may be beneficial.

CONCLUSIONS

Predicting which analytical strategy will lead to the next generation of rapid, sensitive, specific, and low-cost microbiological assays is difficult. Microbiological assays in different fields of application are converging toward analytical specifications that may have a common techno logical solution. Some new or improved analytical technologies have potential for development as microbiological assays. Immunosensors and more sensitive methods of signal generation and background reduction for immunological assays offer improved detection limits in sandwich assays for detecting microbial antigens. Microchip and biochip devices offer some unique possibilities for sample preparation and integrated analysis, and the newer single-molecule detection methods provide the potential level of sensitivity demanded in some microbiological assay applications.

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Afterword

It has taken a few years to bring this book from conception to publication, and it is interesting to reflect on the developments in technology, acceptability, and attitude that have occurred in the interim.

ADVANCES IN TECHNOLOGY

Improvements in technology have continued, resulting in the appearance of new or modified detection methods for microorganisms that may not have been covered within the contents of the book. All of these technologies and methods have been developed and evaluated for application within the food and clinical industries. None has been validated or adopted for industrial pharmaceutical applications.

MicroFoss system (Foss Electric, Denmark) and Biosys (Remel, USA) are detection systems based on classical cultural methods such as color change and redox potential. The results that are generated give a time to detection that is similar to that of impedance. They are able to enumerate microorganisms and correlates well with plate counts. They also claim to detect specific groups of microbes such as coliform bacteria and yeasts, as well as specific organisms such as *Salmonella* and *Listeria.*

Detection methods using microscopy can yield very rapid results but they usually require extensive sample preparation and a skilled operator, and, as such, these methods are usually labor intensive, have low sample throughput, and poor sensitivity. However, developments in confocal microscopy may extend the scope of this oldest of detection methods. Confocal microscopy can be applied to samples without extensive preparation processes, thus avoiding artifacts, and observations can be made in three dimensions without physically sectioning the specimen. It can be used to study the location, attachment, viability, and survival of microbes (see Takeuchi and Frank 2001). This technology is in its infancy and is currently used for research application in the food industry.

The Cellfacts system (Microbial Systems Ltd, UK) uses the well-established Coulter Counter principles of particle detection to detect microbes in clinical and food samples. The method generates information about both the number and size of particles that is directly related to their metabolic state. The system provides a novel and effective quality control methods for industrial fermentation processes to optimize yield and performance. Incorporating specific metabolic markers is also claimed to provide a method for the detection of specific organisms such as *Salmonella.* The system has not been evaluated for pharmaceutical applications.

Immuno-magnetic particles have been used for novel applications or in conjunction with other technologies to produce new detection methods. Origin, produced by IGEN International Inc. (USA and UK), has coupled very small ferro-particles with a ruthidium ion to provide a novel end detection system for pathogens such as *Salmonella.* Matrix Technology (UK) has used immuno-magnetic beads to separate and concentrate pathogens during conventional enrichment procedures. This selective capture process reduces the time and labor required for sample preparation and enrichment, and automatically increases the number of available target organisms, reduces the number of interfering organisms, and thus increases the probability of detection of specific organisms, e.g., *Salmonella.* The technology has been validated for food samples but not for pharmaceutical applications.

Alternative detection systems using the specificity of bacteriophages have also been developed. Alaska Diagnostics (UK) use phage-specific lysis coupled to enhanced luminescence by the detection of adenylate kinase to detect pathogens. The technology is being commercialized for food applications. BioTec Ltd. (UK) uses phage lysis in a more conventional plague-type agar plate assay to detect broad groups or indicator organisms and possibly even specific pathogens. Some research has

been targeted at objectionable organisms in the healthcare sector but the technology has not been validated or commercialized. BIND (BioControl [formerly IDEXX], USA) uses genetically engineered phage carrying the ice nucleation genes to produce a novel end detection system for pathogens such as *Salmonella.* The technology has been validated and approved for food applications only.

Automation in DNA analysis has been established (Cockerill and Smith 2002). PCR is set to revolutionize the clinical market by reducing the time and cost for the detection of Group A Streptococci in throat swabs. DNA-based detection systems are becoming established for the identification of microbes as well as the detection of specific organisms. Very rapid identification has also been shown possible by the use of powerful physico-chemical techniques such as MALDI-TOF Mass Spectroscopy. Whole untreated bacterial cells of USP objectional organisms can be differentiated and identified using cluster analysis performed on the relative intensity and mass of 8 to 20 major peaks from their spectral fingerprints.

ACCEPTABILITY

Significant advancement has been made in establishing the criteria for evaluating and validating alternative microbiological methods. An all-party industry Task Force led to the publication of the PDA Technical Report 33 (Bauer et al. 2000), and more recently the USP published as a draft a similar guidance document for the General Information Chapter <1223> (Anon 2002).

The acid test for the acceptance of any alternative method is its recognition and acceptance by regulator agencies as part of Market Authorization for licensed pharmaceutical products. The first and only method to receive this accolade to date is ATP Bioluminescence for nonsterile products. Both the validation process and the regulatory review process were simple and easy with both user and supplier working together to generate the necessary support information. Interestingly, this is a presence and absence test that is replacing a Microbial Limits Test for products with little or no expected bioburden. This is a clear example where the new alternative method is superior to the conventional method that is itself no longer fit for the purpose.

ATTITUDE

Experience has shown that there are no obstacles to the implementation of alternative microbiological methods. Everyone wants better methods that deliver improvements in detection speed, capability, and reliability to ensure the safety of pharmaceutical products. However, improvements, not just simpler, faster, more sensitive methods targeted at end product screening, need to come from several sources. We need a more holistic pragmatic approach that includes a needs assessment based on actual hazard and risk and the practicalities of sampling and testing statistics. That is, we need a modern day system based quality assurance along the whole manufacturing process.

The rate of implementation has been slow due to number of factors including the inherent inertia in the system and a fear of what the regulators may say. Regulators themselves are not immune to change, and many recognize the need for change and want to see the use of best available technology. However, inspectors have to enforce a wide range of standards, and many have only limited microbiological knowledge and experience. It is therefore unreasonable to expect them to be experts in everything. Many industrial microbiologists carry great responsibility but are rarely invested with the appropriate authority. Consequently, they fail to recognize their own strengths and are often forced to blindly follow the established prescriptive procedure, conveniently avoiding consideration of the real practical problems of industrial microbiology. Hence, both industrial microbiologists and regulators adopt the path of least resistance by following the pharmacopoeial guidelines that are intended as a common reference point and include methods based on historical custom and practice. Accordingly, the status quo is the continued use of outdated methods.

In August 2002 the FDA announced its intent to merge science-based risk management with an integrated quality systems approach to focus the agency's cGMP requirements more squarely on potential risks to public health. This initiative will integrate the most current quality systems and risk management approaches and will encourage the adoption of modern and innovative manufacturing technology. It includes such innovations as regulatory process changes to encourage manufacturing innovations, emphasize a risk-based approach to quality control, and enhance key aspects of FDA inspections.

There are several systems for the assessment and management of risk that cover complex manufacturing processes. The identification of the real (and not perceived) hazards is an essential component and foundation of all risk management systems. Accordingly the outcome of any new risk management strategies and procedures must include a rational reassessment of the real microbiological hazards, including appropriate, practical analytical methods to monitor microbiological quality control and ensure safety.

Clearly there is no shortage of ideas and technologies for improved or alternative methods, only the will to make the change for the better. Opinion leaders and influencers in the industry are preparing the ground for change and improved methodology. The desired revolution will occur only if we all have the courage and determination to work together to challenge the status quo in a nonadversarial environment to make it happen. Failure to do so will result in another 100 years

blindly following and complaining about out-dated methods that are no longer fit for the purpose in the ultraclean manufacturing environment of the twenty-first century.

You do not get what you deserve—you get what you negotiate!

Martin C.Easter
Anthony M.Cundell
January 2003

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Dr. Martin Easter is General Manager at Hygenia International Ltd., which provides rapid and convenient testing devices for microbiology methods. He was educated in the United Kingdom, where he received his first degree in applied biology (specializing in microbiology and bio-chemistry) and his higher degree in microbial biochemistry. During his formative years, Dr. Easter developed an interest in alternative and rapid methods. As Director of Scientific and Regulatory Affairs at Celsis Ltd., he led the validation and approval team implementing ATP bioluminescence methods in the pharmaceutical and healthcare industries. Now recognized as an expert in alternative methods and systems for microbial safety and QA, he has developed a diagnostic media for use as a rapid test for *Salmonella,* using impedance, which has achieved AOAC Final Action Approval status. Dr. Easter was instrumental in initiating the PDA Task Force on rapid and alternative methods and has published widely.

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Dr. Tony Cundell currently directs a small corporate microbiological development and statistics group serving all the domestic manufacturing sites of Wyeth Pharmaceuticals. He has graduate degrees in biochemistry and microbiology from the Victoria University of Wellington and Lincoln University in New Zealand. After his post-doctoral studies in environmental microbiology at the University of Rhode Island and Harvard University, he spent 25 years working in Quality Control in the medical device, biologics, and pharmaceutical industries. Dr. Cundell was a member of the PhRMA Task Force on Environmental Monitoring in Non-Sterile Production Areas and chaired the PDA Task Force on the Evaluation, Validation, and Implementation of New Microbiological Testing Methods that resulted in the development of PDA Technical Report #33 published in July 2000. Subjects of recent publications are environmental monitoring, stream sterilization, and validation of rapid microbial enumeration methods. Most recently he was appointed to the 2000–2005 USP Microbiology Committee of Experts.

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Dr. Edward Fitzgerald has 30 years' experience in the quality control testing of biological products and their related regulatory aspects. He has worked for the Center for Biologics Evaluation and Research (CBER) and the Food and Drug Administration and its predecessor organizations since 1967. During that time, Dr. Fitzgerald was appointed Deputy Director (1974) and Director (1990) of the Division of Product Quality Control, CBER, FDA. Following his retirement in 1997, he formed Fitzgerald Consulting to provide regulatory and technical advice for biopharmaceutical firms regulated by FDA.

Dr. Fitzgerald received his BS degree in biology from Georgetown University and his PhD in microbiology from Catholic University. He has been a member of the U.S. Pharmacopeia Committee of Revision, now called the USP Council of Experts (COE), since 1980 and was recently elected to his fifth term (2000–2005). He is currently assigned to the Analytical Microbiology subcommittee of the COE. Dr. Fitzgerald is also a member of the International Association for Biologicals, the American Society for Microbiology, and the PDA.

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Dr. H.Donald Hochstein obtained a Doctor of Public Health degree in 1970. He worked at the NIH as a microbiologist from 1958–1972, when the FDA replaced the NIH. Dr. Hochstein remained with the FDA's Bureau of Biologics, where he was responsible for all aspects of LAL, until his retirement in 1997. He has over 50 scientific publications.

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Kirsty Wills is a consultant, handling varied technical marketing assignments. She has a degree in microbiology and was previously employed by Celsis. Ms. Wills has played a key role in gaining acceptance for rapid methods in pharmaceutical microbiology. She has presented ATP bio-luminescence technology to the FDA and many other regulatory agencies. As a member of the PDA Task Force that wrote Technical Report #33, she helped to pioneer change in pharmaceutical microbiology validation.

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