Molecular Methods *for the* Detection *and* Characterization *of* Foodborne *and* Environmental Pathogens

Suresh D. Pillai, Ph.D.
Professor of Microbiology and Texas A&M AgriLife Faculty Fellow & Director
Departments of Poultry Science and Nutrition and Food Science
Director, National Center for Electron Beam Research—an IAEA Collaborating Centre for Electron Beam Technology, Texas A&M University

Jessica A. McKelvey, M.S.
Research Associate, Food Safety and Environmental Microbiology Program
Department of Poultry Science, Texas A&M University

DES*tech* Publications, Inc.

Molecular Methods for the Detection and Characterization of Foodborne and Environmental Pathogens

DEStech Publications, Inc.
439 North Duke Street
Lancaster, Pennsylvania 17602 U.S.A.

Printed in the United States of America
10 9 8 7 6 5 4 3 2 1

Main entry under title:
Molecular Methods for the Detection and Characterization of Foodborne and Environmental Pathogens

A DEStech Publications book
Bibliography: p.
Includes index p. 151

Library of Congress Control Number: 2016959347
ISBN No. 978-1-60595-079-2

Contents

Preface

DETECTING and characterizing microorganisms are critical to meet today's public health, environmental microbiology, and industrial microbiology needs. However, without an accurate representative sample, detection and characterization methods are only of very limited value. Microbial detection and characterization techniques are growing at a breathtaking pace, thanks to accelerating advances in omics technologies and bioinformatics. In spite of these advances, the underlying principles of sampling, sample processing, and sample concentration are still relevant. For example, conventional culture media-based methods such as Colilert and most-probable-number (MPN) methods are still very relevant, especially when it comes to meeting regulatory benchmarks. Nevertheless, in regulatory agencies—especially in the developed countries, such as the U.S. Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA)—there is a growing demand for molecular biology-based methods.

Today, most students acquire the skills to perform molecular analytical skills through independent laboratory research. Very few textbooks targeting senior undergraduate and graduate-level students provide the theory and step-by-step protocols for these molecular methods. This textbook is an attempt to address this unmet need. This textbook, targeted to senior undergraduate and graduate-level students will be suitable for laboratory courses that deal with microbial detection and characterization.

The content of this book is based on a graduate-level course titled "Molecular Methods of Microbial Detection and Characterization"

that Professor Pillai teaches at Texas A&M University. Food science, nutrition, animal science, poultry science, soil science, oceanography, entomology, veterinary school, medical school, and engineering graduate students have enrolled in this course over the past 15 years. We acknowledge that sophisticated laboratory instruments are required for many of the methods described in this book. However, we trust that most colleges and universities would have most of these instruments on their campuses. If this book is adopted as a course textbook, not all chapters may be suitable for the course. However, we are confident there is enough material in this book that instructors will be able to design a comprehensive laboratory course to train the future generation of microbiology professionals. There is a need for microbiologists who are conversant with contemporary technologies and who are trained in these contemporary laboratory techniques. We hope this book meets this need.

Acknowledgements

To all my students and team members past and present who, by using molecular techniques in their research, have made my laboratory *the* place to learn how to use molecular methods for microbial detection and characterization. A BIG thank you to Melinda and Eric for their humor and support all along the way and to Joseph Eckenrode—you have been patient and understanding while we worked on this book. Your e-mails brought forth both guilt and motivation! Fortunately, motivation trumped guilt! Thanks for your strong support along the way.

SURESH PILLAI

A special thanks to Chandni Praveen and Charlotte Hieke, previous graduate students at Texas A&M University, who assisted with the laboratory designs and protocols.

JESSICA MCKELVEY

CHAPTER 1

Microorganisms: Detection and Characterization

1.1. MICROBES ON EARTH

By modern estimates, Earth is approximately 4.5 billion years old (Jacobsen, 2003). Microbes have had a much longer history than humans have on this planet. Microbes have been here for almost 3.8 billion years, while humans have been on Earth for just a minuscule fraction of that time. Figure 1.1 is a schematic representation of Earth's age as a 12-month calendar. Microbes started colonizing the earth since February while humans only since around 10:00 P.M., December 31.

Understanding the age of microorganisms on Earth is critically important when we attempt to detect, isolate, and characterize microorganisms in their natural habitats. During their origins on earth, microorganisms have experienced rather strong perturbations in terms of temperature, gaseous conditions, tectonic movements, volcanic conditions, and meteorite impacts. They have withstood strong ionizing radiation conditions prior to the formation of the atmosphere. Thus, microorganisms have developed and perfected extremely efficient adaptive and survival mechanisms in the natural environment. Their genetic plasticity, genetic diversity, and numbers attest to how successful they are in terms of surviving and adapting to changing conditions on this planet. Almost every single spot on Earth has been colonized. Only recently, with the advent of advanced molecular and imaging tools, have we come to appreciate the breadth and depth of microbial colonization of natural and manmade ecosystems.

Microorganisms have developed strong survival mechanisms to withstand environmental conditions. Their morphological, genetic, and physi-

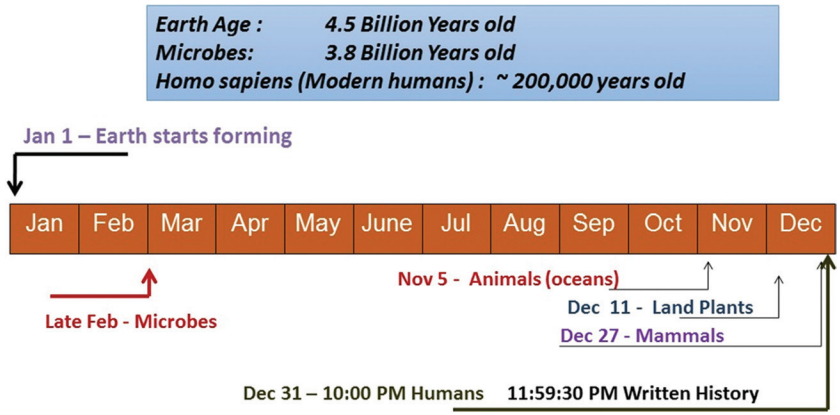

FIGURE 1.1. Schematic Representation of Earth's Age as a 12-Month Calendar.

ological adaptions include the circularization of the bacterial cells during stress conditions, the accumulation of nutrient storage molecules during periods of starvation and the switching on of stress response genes when exposed to stress conditions. Today, we know that microbes outnumber humans on this planet by unimaginable numbers. The human population is approximately 7.4 billion (or, in other words, 7.4×10^9 humans). There are more microorganisms than this number in just one gram of human feces! Even on a single human body, microbes are thought to outnumber human cells by a factor of 10. Thus, it is not surprising that microbes continually influence human and animal health. Many of these microbial populations are opportunistic pathogens and under the right circumstances proliferate and cause disease (ILSI, 1996). There are a number of excellent books and review articles that detail the nuances between opportunistic pathogens, commensals, and frank pathogens (Abt and Artis, 2013; Behar and Louzoun, 2015; Packey and Sartor, 2009).

1.2. MICROBES, HUMANS, AND ANIMAL HEALTH

Given the close association between microorganisms and their human and animal hosts, it is not surprising that microorganisms are now being identified as the etiological agent for a number of human illnesses ranging from diarrhea to Type 2 diabetes, as well as behavioral disorders. Domesticated and companion animals are also thoroughly colonized by a variety of microbial populations. Many of these microorganisms are responsible for a number of animal diseases in both domesticated spe-

cies and wildlife. A variety of zoonotic diseases associated with pathogens, such as the Ebola virus, avian influenza virus, and *Salmonella* spp., are known to be transmitted to humans by animals. The food industry relies very heavily on poultry, swine, and cattle industries. These industries are challenged by the repeated occurrence of foodborne pathogen outbreaks often associated with these animals. Human-to-human transmission of pathogens also occurs routinely. This includes some of the highly publicized outbreaks such as cholera, typhoid, and sexually transmitted diseases. Quite often, the pathogens that are transmitted between humans or between animals and humans involve either an insect vector (such as Zika virus or malaria) or persistence (and possibly multiplication) in the environment. The role of fomites such as doorknobs, towels, and so on are also key factors controlling the spread of microbial pathogens that are responsible for a number of infectious diseases. A majority of the highly publicized outbreaks today are associated through a pathogen exposure via the environment or fomites (door handles, curtains, surfaces, etc.) example outbreaks associated with Shiga toxin producing *E. coli*, rotavirus, norovirus, *Listeria* sp., etc. Other than infections transmitted directly between humans via the reproductive tract or by exchange of bodily fluids, it would be a challenge to name an infection that did not involve either aerosols, water, soils, food, or fomites. Table 1.1 is a list of selected pathogens that are known to be associated with exposure to the agent either via the environment or through contaminated foods.

Given the importance of identifying environments that harbor infectious organisms and quantifying what the potential exposure is, it is imperative that public health protection programs around the world have robust public health laboratory capabilities as well as trained individuals to detect, isolate, and characterize microbial pathogens. At the very least, these programs should have the capabilities to detect and characterize microorganisms (pathogens as well as nonpathogens) from natural and man-made environments. To deal with the challenges of the growing global population, the frequency with which people travel around the world, and the globalization of food supplies and food consumption, it is also becoming imperative that public health programs be able to accurately and rapidly "relate" organisms that are isolated from different parts of the world as well as determine the virulence potential and compare the expression of virulence genes from different isolates. These requirements necessitate the use of molecular detection and characterization technologies.

TABLE 1.1. Selected List of Public Health Relevant Pathogens, Known Reservoirs, Transmission Routes, and Typical Symptoms.

Organism	Normal Host/Reservoir	Transmission Route	Disease Caused/Symptoms
Bacteria			
Salmonella typhi	Humans	Food, water	Typhoid fever
Salmonella enteritidis	Chickens, cattle	Food, water, aerosols	Gastroenteritis
Listeria monocytogenes	Environment, animals, humans, vegetation	Food, fresh produce, ready-to-eat (RTE) foods	Listeriosis
Campylobacter jejuni	Chickens	Food, water	Gastroenteritis
Clostridium perfringens	Soils, companion animals, processed foods	Food, vegetables	Clostridial food poisoning
Shiga toxin producing *E. coli*	Humans, cattle	Water, food	Gastroenteritis, diarrhea
Protozoa			
Cryptosporidium parvum	Environment, variety of mammals, cattle,	Water, food, aerosols	Acute gastroenteritis
Cyclospora cayetanensis	Domestic animals, poultry, birds	Water, food, soil	Diarrhea, abdominal cramps
Viruses			
Norovirus	Humans, animals	Person-to-person, food, surfaces	Diarrhea, vomiting, nausea, stomach pain
Rotavirus	Humans, animals	Fecal-oral route, hands, food, water, surfaces	Watery diarrhea, vomiting, abdominal pain
Hepatitis A virus	Humans	Fecal oral route, food, water, drinks, surfaces	Fever, fatigue, nausea, vomiting, dark urine, jaundice

1.3. HISTORY OF MICROBIAL DETECTION AND CHARACTERIZATION

Humans have always been inquisitive. The desire to "look at" microorganisms fueled the development of the early microscopes in the early seventeenth century. This same inquisitiveness is still within us. Whether we send robotic missions to planets such as Mars or to the deep oceans, we always outfit them with sophisticated optics, sensors, and analyzers. These tools have literally opened our eyes to the world of microorganisms in which we live. The same need or desire still lingers the twenty-first century. The scientific questions we ask today are quite different from what we were after even five years ago. Today, it is no longer sufficient to know if we are exposed to pathogens in the foods we eat or the water we drink. We want to know the types, and numbers of fecal origin bacteria, viruses, and protozoa. Not only are we content with knowing that fresh produce contains noroviruses; we are curious to know whether they are of the GII genotype, and if they are of the same genotype that was isolated overseas months or even years ago! The history of microbial detection and characterization can be broadly divided into three periods namely:

1. *Pre-PCR period*: characterized by development of sampling methods, isolation media, enrichment media, chromogenic media, biological viability assays, fluorescent dyes, and more. These developments resulted in better sanitation programs and ultimately have led to humans making major strides in our public health protection programs.
2. *Post-PCR period*: The development of the polymerase chain reaction (PCR) nucleic acid amplification assay in the late 1980s was, in many ways, akin to the development of the steam locomotive. The invention of this molecular biology assay resulted in the inventor, Kary Mullis, receiving the Noble Prize in Chemistry. The method was simplistic in concept but thoroughly groundbreaking in its applications and the information it provides. Coincidentally, the author (SDP) was fortunate to be in graduate school at this time in history and was among the very first to demonstrate the use of the PCR amplification assay to detect specific organisms such as fecal coliforms in soils and sediments (Josephson *et al.*, 1991).
3. *Post-deep sequencing period*: The development of high-through-

put, massively parallel sequencing methods and instrument platforms, such as pyrosequencing, and MiSeq, have opened up significant opportunities to identify the entire microbial diversity (or close to it) of almost any sample. The findings from these types of analyses are truly revolutionizing our understanding of the "microbiome" that we and our plant and animal neighbors harbor.

In our estimation the changes from these molecular tools have already or have the potential to completely rewrite our understanding of the role of microbes in our day-to-day existence.

1.4. BASIC MICROBIOLOGY TECHNIQUES

There are virtually hundreds of excellent review articles, books, and book chapters that deal with the different conventional methods available for microbial detection and characterization. Conventional methods in our parlance are those methods that mostly rely on the use of viability or culturability of the target organism for detection, quantification, and characterization. One could argue that microscopic methods would fall into the realm of conventional methods. However, without the use of specialized dyes, such as specific fluorescently labeled antibodies, it would be impossible to accurately identify the target organism(s). Some of the classic usage of this method has been the detection of pathogens such as *Cryptosporidium* spp. and *Toxoplasma* spp. in soils (Orlofsky *et al.*, 2013; dos Santos *et al.*, 2010). Significant advances in microscope-based detection and characterization of target organisms have also occurred with the development of *in-situ* hybridization employing fluorescent probes (Dinis *et al.*, 2011).

Culture-based methods have been the cornerstone of public health and medical microbiology for several decades. In clinical laboratories, without the actual isolation of a suspect pathogen in a clinical laboratory, the pathologists would never attribute an illness to a microbial etiology. Those days of absolute reliance on culture-based methods have fortunately passed. The use of culture-based methods to detect pathogens has some positive attributes. For one, it would suggest that the suspect organism is viable. Secondly, it allows for the enumeration of actual number of viable organisms. Additionally, the ability to isolate the organism in a viable and culturable state facilitates using a variety of biochemical reagents for characterization. Also, the availability of live cultures provides for rather strong forensic evidence, which is often

the necessity for criminal cases involving food poisoning, deliberate contamination, and so on.

In a patient who is exhibiting overt symptoms, the pathogen loads in his or her body are generally considered high. Very often they can be as high as 10^5 to 10^6 organisms per sample volume. This high titer makes it convenient to isolate these organisms using just a throat or rectal swab. The high pathogen titers will manifest themselves as characteristic colonies on selective media. Selective media are those types of culture media that allow only the growth of a specific type or types of organisms. Selective media involve the use of specific carbon sources, specific biochemical, specific dyes, or specific incubation conditions. Selective media help in the quick identification of the target organism.

In some instances where it has been technically challenging to formulate selective media, the use of differential media has been widely employed. Differential media are those types of media where different target organisms would exhibit differently colored or reacting colonies. Differential media makes use of the differences in the biochemical pathways that exist amongst specific pathogens. For example, generic *E. coli* strains can ferment sorbitol, and therefore these cells form pink colonies on media containing sorbitol. However, shiga-toxin-producing *E. coli* strains (for the most part) do not ferment sorbitol. Colonies of shiga-toxin-producing *E. coli* strains are colorless on media containing sorbitol. Therefore, the strategy of conventional methods is to design a selective media for all *E. coli* such as MacConkey agar. All *E. coli* strains have a characteristic colony color on this medium. Therefore, in this regard, MacConkey agar can be termed a differential medium (since it does not totally prevent other bacteria from growing on them). However, if sorbitol is added as the carbon source to MacConkey agar, only *E. coli* cells will grow on this medium. In this case, it now becomes a selective media. All generic *E. coli* strains will form pink colonies on MacConkey-sorbitol agar media while shiga-toxin-producing *E. coli* strains will be colorless.

In samples where the initial pathogen titers may be low, the use of enrichment methods has facilitated the detection of organisms from such environments. Enrichment media help to selectively or differentially allow the proliferation of the target organism from samples. Such media are generally liquid culture media, for example, lactose broth (to enrich lactose-fermenting organisms). The enrichment media is then followed by the use of differential and/or selective media. The drink-

ing water industry and the food industry historically used enrichment media for detecting fecal contamination and specific pathogens. The major drawback of enrichment media is that the results are not quantitative in that it is impossible to accurately estimate the starting number of organisms from an enrichment culture. However, microbiologists have figured out the use of statistically based enrichment media protocols to estimate a most probable number (MPN) of starting organisms from enrichment media results. The MPN is the cornerstone of the EPA methods to detect fecal coliforms, total coliforms, *E. coli*, *Enterococci*, and *Salmonella* from environmental samples. MPN methods are robust but cumbersome since they involve a large number of tubes, media, and expertise. The U.S. FDA has also started using MPN-based approaches to quantify starting levels of specific pathogens, such as *Salmonella* spp. and *Listeria* spp.

One attribute of conventional methods is that the level of microbiology technical skills required to use such methods is low. Also, most of the materials to prepare and use conventional microbiology methods are also relatively inexpensive. This automatically translates into a lower cost per test.

1.5. CHALLENGES ASSOCIATED WITH CONVENTIONAL MICROBIOLOGY TECHNIQUES

Pathogen detection and characterization approaches that rely on conventional methods involving the culturing of organisms are beset with a number of shortcomings. The biggest drawback is that it is now well established that less than 1 percent of the microbial populations found in nature are able to be grown on culture media. Under environmental conditions many of the pathogens that cause a variety of human infections are in a state called viable but nonculturable (VBNC) (Wu *et al.*, 2016; Zhong *et al.*, 2016). Interestingly, there are a number of books and review articles that describe this microbial state in terms of microbial morphology, physiology, genetics, and metabolism. Briefly put, the VBNC state refers to the inability of organisms isolated from the environment to grow on typical laboratory culture. Thus, one can expect the possibility of environmental microbiologists not detecting the presence of such pathogens if traditional culture media is the only method employed.

A variety of explanations have been put forth to explain why environmental isolation attempts fail. One of the most widely cited reasons

is that microbes in the environment are in a "survival" mode because of the limited availability of moisture and nutrients. The survival strategies that microbes develop are indeed quite spectacular (Roszak and Colwell, 1987). Typical enrichment, selective, and differential media have significantly excess amount of nutrients compared to what microbes encounter in the natural environment. Thus, when environmental samples are plated on culture media, these stressed cells are so physiologically shocked by the excess amount of nutrients that they fail to grow and multiply. The inability to accurately estimate the actual number of target organisms from environmental isolations because of the VBNC state of these organisms is one of the major drawbacks of culture-based methods.

Another challenge associated with the use of culture media is the choice of media that the analyst has to utilize to screen for human pathogens from different samples. For example, in a case of suspected sewage intrusion into a drinking water distribution system, which target organism(s) should the diagnostic laboratory test for? Which specific or differential media should the lab utilize? These are major tactical decisions and potentially life-and-death decisions. Imagine a situation where there is a suspicion of deliberate contamination by multiple pathogens of food or drinking water supplies. Imagine you are the head of the public health laboratory and you know that the death toll from this terrorist act is mounting by the hour and you have to make quick decisions on what to test for and the type of culture media that needs to be used. Wouldn't it be beneficial to have the choice of one or more methods that allow you to screen for a large number of bacterial, viral, and protozoan pathogens rather quickly?

Time is money and wasted time can also be associated with possible fatalities when it comes to the diagnostic microbiology laboratory. Culture methods take anywhere from eight hours to as long as a week to obtain a confirmed detection of some target pathogens. Viral pathogens take multiple weeks to culture to obtain results. Thus, the time it takes for incubation of culture media and the time it takes to confirm the identity of the suspected organism are inordinately too long for present-day needs. In clinical samples, since the numbers of target organisms are generally higher than environmentally associated pathogens, clinical diagnostic labs can get away with a shortened time between sampling and detection/confirmation.

Another major shortcoming of culture-based methods of pathogen detection is the issue of detection specificity. Detection specificity re-

fers to the ability to discern the difference between closely related bacterial genera, bacterial species, or bacterial strains. Thus, can a culture-based method differentiate between *Citrobacter* spp. and *Salmonella* spp.? It is important to bear in mind that selective and differential media were designed with custom formulation of carbon sources, diagnostic dyes, and so on, based on taxonomic differences between the different organisms. However, taxonomic changes do occur with our improved understanding of the different organisms. Therefore, bacterial genera and species are often reclassified, and often culture media are unable to keep up with these slight changes in the taxonomy of the organisms.

In addition to VBNC and time considerations, some pathogens are unable to be cultured by any known laboratory method. One such example was the human norovirus. The ability to culture these viruses in the laboratory was one of the most elusive of all microbiological methods. There has been a recent report of this virus being cultured in the laboratory.

1.6. MOLECULAR DETECTION OF MICROORGANISMS

The advent of PCR amplification assays and high-throughput deep microbial sequencing tools have opened up completely new approaches to detecting and characterizing microbial populations. Once again, there are numerous books and book chapters that deal with the different molecular tools that are available for the clinical, food, and water industries. With molecular tools such as DNA:DNA probe hybridizations, PCR assays, DNA fingerprinting, and deep sequencing, it is now possible to detect the presence/absence of multiple target organisms extremely fast.

There are reports of assays that can provide yes/no answers for virtually hundreds of different organisms in a few hours. The use of molecular tools permits a variety of different questions to be asked. These can range from the identity of the organism, the genetic relatedness of organisms, the presence or absence of specific virulence attributes as well as the expression of one or more different virulence genes. The specter of biological weapons being used by Iraq during the first Gulf War in 1991 and the events of September 11, 2001, followed by the anthrax attack on the U.S. Senate later that year, spurred significant investment by the government to develop rapid molecular biology-based detection tools. The technologies that were and are being developed are truly spectacular. Many of them have a dual use capability in that the

core technology can used for detecting pathogens in the clinical laboratory for public health purposes as well as to detect deliberate biothreat agent attack for terroristic attacks. This textbook will provide specific details on some of these methods.

1.7. DETECTION SPECIFICITY AND DETECTION SENSITIVITY

The concepts of detection specificity and detection sensitivity are extremely important when it comes to the detection of pathogens, especially in the context of molecular methods. Detection specificity can be broadly defined (as mentioned earlier) as the ability to discern the difference between closely related bacterial genera, bacterial species, or bacterial strains. This is no easy task because in molecular assays the differences between organisms are based on genetic-level differences. The organisms are differentiated based on differences in one or more different regions of their genomes. This by default means that the genetic information about the different organisms has to be known *a priori*. The significant advances in instrumentation and bioinformatics tools that took place during the human genome sequencing, coupled with the spectacular developments of high-throughput, massively parallel sequencing methods and platforms over the past five years, have completely revolutionized our ability to obtain the complete sequence information of an organism. Today, that information, either in pure culture or present in a microbial consortium, can be obtained in about an hour. What takes the largest chunk of time is the bioinformatics data analysis that is performed downstream of the actual sequencing protocol. There are excellent resources on some of these techniques and tools. So, today it is possible to obtain high-resolution nucleic acid sequence information about different microbial isolates.

Detection sensitivity refers to the ability to detect the smallest number of target organism(s) from a unit amount of sample. Detection sensitivity, especially when discussing molecular methods, cannot be divorced from a discussion of sampling. Sampling, to be used in conjunction with either traditional culture-based methods or molecular methods, is extremely critical in terms of the overall detection sensitivity. Intuitively, the larger the sample analyzed, the greater the detection sensitivity. This is based on the assumption that it is possible to adequately process the sample to the required volume, which is then used in its entirety in the molecular assay. However, this never happens in reality. Molecular

assays often involve a variety of different reagents, and the entire volume of each assay is generally no more than 100 μL. Often the typical volumes are in the 25 μL to 50 μL range. Out of this volume, only 10 μL is set aside for the actual sample. The rest is comprised of assay reagents and assay buffers.

Let us assume that we are interested in detecting a fecal pathogen such as *Shigella* sp. in drinking water. Do we sample 1,000 L or 100 L or 10 L or 1 L or 100 mL or 1 mL? We know by now that using a larger volume improves the detection sensitivity. So if we collect 1,000 L of the drinking water sample, how do we use it in a molecular assay? Do we just pipette out 10 μL from the collected sample and use it in the assay? If this is the case, why couldn't we just collect 10 μL directly from the drinking water distribution line and use it in the assay? So, the technical challenge is how do we "stuff" 1,000 L or a loaf of bread, or 100 acres of a field, or tanker truck of raw milk into a molecular assay? Thus, when we discuss molecular assays, the issue of sample processing is of utmost importance. (In culture-based methods as well, sample processing was not paid close attention, and the usual routine is to spread plate 0.1 mL of the sample directly on a plate.)

Sample processing includes one or more laboratory-based protocols that are used to prepare the original sample to a volume and format suitable for use in a molecular assay. For example, if the objective is to detect biothreat agents in bioaerosols, how do we sample and process the sample for molecular assays? There are different methods of sample processing, and some of them will be discussed in subsequent chapters. There are also excellent review articles on sample processing as it pertains to molecular detection of pathogens.

Back to the question of detection sensitivity and sample processing for detecting *Shigella* sp. in drinking water. Let us assume that we concentrate the 1,000 L to 10 mL by a cartridge filter (for example, IDEXX Filta-Max filter). Let us also assume that we then concentrate the 10 mL sample down to 50 μL by use of a commercial concentrator (e.g., Amicon). The 50 μL concentrated volume is a suitable volume from which the 10 μL can be aliquoted out for the molecular assay. Theoretically, all 50 μL of the concentrated sample can be used in separate molecular assays; that is, five 10 μL aliquots in five molecular assays. The results from the five molecular assays will provide the answer to the original question as to whether the 1,000 L of the drinking water sample contained the target pathogen, *Shigella* sp.

In the example above, it is important to differentiate between *assay*

sensitivity and *method sensitivity*. Assay sensitivity refers to the least number of target nucleic acids sequences (that is, copy numbers) that must be present in a single assay for a reliable detection. As mentioned earlier, if the molecular assay involved the use of 10 µL of the original sample, and if the assay is able to detect the presence of one target gene copy, the *assay sensitivity* is termed as one target gene copy (from 10 µL sample volume). If there is only one gene copy in *Shigella* sp., then the detection sensitivity can be described as one *Shigella* sp. cell. By using this information, it is then possible to back-calculate the *method sensitivity*. Using the example provided above, if all five molecular assays gave positive signals, then it assumes that each assay contained at least one *Shigella* sp. cell. Therefore, the 50 µL contained five *Shigella* cells. Since the 50 µL originated from 1,000 L, the method sensitivity (based on this protocol) is five *Shigella* cells.

It must be emphasized that how the original sample is processed and what fraction of the original sample is actually analyzed by the method dictates the ultimate method detection sensitivity. It is important to understand the difference between assay sensitivity and method detection sensitivity. It is not a given that molecular assays will always be more sensitive than traditional culture methods. Let us assume that we are interested in detecting male-specific coliphages (a fecal indicator) in groundwater. The EPA sampling protocol for male-specific coliphages calls for sampling 100 mL. The EPA culture-based protocol calls for detecting coliphages in the 100 mL sample directly. Let us assume the EPA protocol can detect one coliphage in this 100 mL sample. Coliphages are detected based on plaques that form on host bacterial lawns. Therefore, the method detection sensitivity for this EPA protocol is one plaque-forming unit (PFU) per 100 mL. Assume we are interested in screening for the coliphages using a molecular assay. Since not all 100 mL can be used in a molecular assay, let us assume we concentrate the sample to 50 µL. If we use the entire 50 µL concentrate (similar to the *Shigella* example above) in one or more molecular assays (and the assay can detect sequences from one virus particle), then the method detection sensitivity of this molecular assay will be similar to the EPA culture-based method (one PFU per 100 mL). However, if the assay requires a minimum of two virus particles in the 10 µL aliquot for detection, then the method detection sensitivity drops to two PFU per 100 mL. In this case the molecular assay is not as sensitive compared to the culture-based phage assay.

Very rarely are molecular assays capable of detecting one target gene

copy (for example, in 10 μL sample volume). Results show that a minimum of between 100 and 1,000 gene copies (in 10 μL sample concentrate) are needed for reliable detection. In this case, it is important to understand the method detection sensitivity. In other words, how many target gene copies are needed in the original sample so that they are detectable in the final aliquot used in the molecular assay.

Figure 1.2 illustrates the concept of assay sensitivity and how molecular assays are at times no more sensitive than culture-based assays. In fact, in the example that is illustrated, if the assay sensitivity is one target organism in 10 μL, this means that there should be at least 100 organisms in 1,000 μL or 1 mL. This in turn suggests that the original sample should contain at least 104 target organisms in 100 mL or 105 organisms in 1,000 mL. If such high titers of target organisms are needed for detection by molecular assays, what is the value of molecular assays compared to culture-based methods? This is where the value proposition of molecular assays in terms of speed, ability to detect a variety of different organisms, independence from the issues of VBNC, and ability to obtain high-resolution information about the organism comes into play. In the example shown in Figure 1.2, detection sensitivity of molecular assays may not be the driver for its application.

It is important to bear in mind that during sample processing there is a very high likelihood that target organisms can be lost due to the inefficiencies of laboratory protocols such as pipetting and centrifugation. Therefore, when establishing method detection sensitivity limits, be aware that simple back calculation of sensitivity from the assay sensitivity may be overestimating the actual detection sensitivity. It cannot be overemphasized that understanding the concept of detection sensitivity is extremely critical when it comes to employing molecular assays.

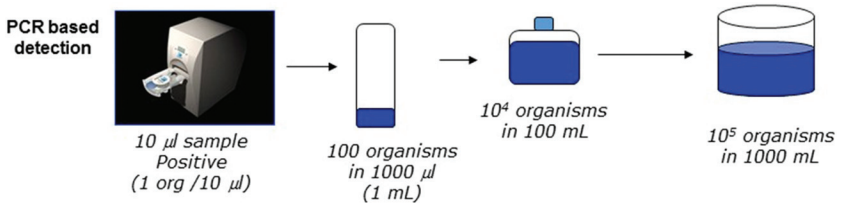

FIGURE 1.2. *Schematic showing the importance of differentiating molecular method sensitivity as compared to overall method sensitivity.*

1.8. COMMERCIAL KITS FOR MICROBIAL DETECTION AND CHARACTERIZATION

As mentioned earlier, there has been significant investment by the government and private industry into the development of molecular methods. This has resulted in the number of commercial kits that are currently available for rapid detection and characterization of microbial pathogens and nonpathogens. One can perform a simple Internet search using terms such as "pathogen detection kits" to see the diverse numbers of manufacturers and types and chemistries that are currently available. There are commercial kits and sampling platforms for obtaining bioaerosol samples, groundwater samples, drinking water samples, food sampling, swabs for sampling surfaces, and kits for sampling human bodies. In addition to sampling kits, there are a number of commercial kits for extracting nucleic acids from a variety of different samples, such as environmental samples, fecal samples, water samples, blood samples, samples from mammalian biopsies, and more. The quality and reproducibility of many such commercial kits are very high. However, it is always the responsibility of the end user to include appropriate controls to ensure that the performance of these commercial kits is as claimed.

1.9. VALIDATION OF COMMERCIAL KITS

Many established commercial kit suppliers in the United States and Europe rely on independent third-party kit validating organizations such as Association of Analytics Communities International (AOAC) and ISO to validate the performance of their kits and methods.

AOAC is a private third-party organization that validates the performance of kits and methods used for detecting pathogens in food, feed, and a variety of other environments. AOAC also publishes "AOAC Official Methods" to provide evaluated methods that can be used by regulatory industries, analytical laboratories and academic institutions. "AOAC Official Methods" are published in the *Official Methods of Analysis of AOAC International* and the *Journal of AOAC International*. AOAC International also manages the Performance Tested Methods (PTM) Program as well as the Performance Tested Methods: Validated Methods Program. AOAC's website (http://AOAC.org) has a portal where there is a listing of all commercial pathogen detection and characterization kits that have received approval as AOAC Performance Tested Methods.

The ISO 16140:2003 defines the general principle for the validation of commercial pathogen detection kits. The standard details the validation protocol and principles of certification. The standard also describes the technical protocols for validation of qualitative and quantitative methods. The standard details the protocols that are required for method comparison studies as well as inter-laboratory validation of protocols. There are third-party entities as MicroVal (https://www.nen.nl/MicroVal-validation/About-MicroVal.htm), which is a European certification organization for the validation and approval of commercial kits used in the food industry that certifies kits per the ISO 16140:2003 standard. MicroVal claims their certification will ensure acceptance by European government inspection labs and commercial labs that are involved in the food trade throughout the European Union.

NordVal (http://nmkl.org) was created in 1999 by the Nordic countries (Denmark, Finland, Iceland, Norway and Sweden) to evaluate and validate the performance of commercial microbiological kits and methods for testing food, animal, water, feed, animal feces and food environmental samples in the Nordic countries.

1.10. REFERENCES

S. B. Jacobsen, "How Old is Planet Earth?" *Science 300* (2003): 1513–1514.

ILSI, "A Conceptual Framework to Assess the Risks of Human Disease Following Exposure to Pathogens," ILSI Risk Science Institute Pathogen Risk Assessment Working Group, *Risk Anal 16* (1996): 841–848.

Evaluation of the role of microbiological criteria—Selection of pathogens.

M. C. Abt and D. Artis, "The Dynamic Influence of Commensal Bacteria on the Immune Response to Pathogens," *Current Opinion in Microbiology 16* (2013): 4–9, doi: 10.1016/j.mib.2012.12.002.

H. Behar and Y. Louzoun, "Commensal Pathogens as a Source of a Coexistence Mechanism," *Journal of Theoretical Biology 370* (2015): 45–52, doi: 10.1016/j.jtbi.2015.01.030.

K. L. Josephson, S. D. Pillai, J. Way, C. P. Gerba, and I. L. Pepper, 1991. Fecal Coliforms in Soil Detected by Polymerase Chain Reaction and DNA-DNA Hybridizations," *Soil Science Society of America Journal 55* (1991): 1326–1332.

C. D. Packey and R. B. Sartor, "Commensal Bacteria, Traditional and Opportunistic Pathogens, Dysbiosis and Bacterial Killing in Inflammatory Bowel Diseases," *Current Opinion in Infectious Diseases 22* (2009): 292–301, doi: 10.1097/QCO.0b013e32832a8a5d.

D. Roszak and R. Colwell, "Survival Strategies of Bacteria in the Natural Environment," *Microbiological Reviews 51* (1987): 365–379.

Ezra Orlofsky, Osnat Gillor, Ann Melli, *et al.*, "Simultaneous Detection of Giardia lam-

blia and Cryptosporidium parvum (Oo)cysts in Soil Using Immunomagnetic Separation and Direct Fluorescent Antibody Staining," *Journal of Microbiological Methods 94*, no. 3 (September 2013), 375–377.

Thais Rabelo dos Santos, Caris Maroni Nunes, Rui Luvizotto, Maria Cecilia, et al. "Detection of Toxoplasma gondii oocysts in environmental samples from public schools," *Veterinary Parasitology 171*, no. 1–2 (July 5, 2010) 53–57.

Jorge M. Dinis, David E. Barton, Jamsheed Ghadiri, *et al.*, "In Search of an Uncultured Human-Associated TM7 Bacterium in the Environment," *PLOS ONE 6*, no. 6 (June 20, 2011), article no. e21280.

Qingping Zhong, Juan Tian, Bin Wang, *et al.*, "PMA Based Real-Time Fluorescent LAMP for Detection of Vibrio parahaemolyticus in Viable but Nonculturable State," *Food Control 63* (May 2016): 230–238.

Bin Wu, Weili Liang, and Kan, Biao, "Growth Phase, Oxygen, Temperature, and Starvation Affect the Development of Viable but Non-culturable State of Vibrio cholerae *Frontiers in Microbiology 7* (March 30, 201): article no. 404.