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Emerging Waterborne Infections: Contributing Factors, Agents, and Detection Tools

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ABSTRACT: Because microorganisms are easily dispersed, display physiological diversity, and tolerate extreme conditions, they are ubiquitous and may contaminate and grow in water. The presence of waterborne enteric pathogens (bacteria, viruses, and protozoa) in domestic water supplies represents a potentially significant human health risk. Even though major outbreaks of waterborne disease are comparatively rare, there is substantial evidence that human enteric pathogens that are frequently present in domestic water supplies are responsible for low-level incidence of waterborne microbial disease. Although these diseases are rarely debilitating to healthy adults for more than a few hours to a few days, enteric pathogens can cause severe illness, even death, for young children, the elderly, or those with compromised immune systems. As the epidemiology of waterborne diseases is changing, there is a growing global public health concern about new and reemerging infectious diseases that are occurring through a complex interaction of social, economic, evolutionary, and ecological factors. New microbial pathogens have emerged, and some have spread worldwide. Alternative testing strategies for waterborne diseases should significantly improve the ability to detect and control the causative pathogenic agents. In this article, we provide an overview of the current state of knowledge of waterborne microbial pathogens, their detection, and the future of new methods in controlling these infectious agents.

KEY WORDS: water, bacteria, protozoa, viruses, emerging pathogens, drinking water, microbiological monitoring.

I. INTRODUCTION

Waterborne microbial diseases, once expected to be eliminated as public health problems, not only remains the leading cause of death worldwide, but the spectrum of disease is expanding and the incidence of many waterborne microbial diseases once thought conquered is increasing.[1,2] These infections, which may be transmitted by contaminated recreational waters, surface water, and ground water intended for drinking, place entire communities at risk. The most common waterborne microbial disease is a mild to acute gastroenteritis illness. Although for most of the population in developed countries minor gastroenteritis may simply mean several hours of discomfort, in developing countries up to 13 million people die every year as a result of the consumption of contaminated water.[3] The symptoms of this condition may be caused by numerous infectious agents, including enteric bacteria, viruses, and protozoa. In general, the viruses are limited to human hosts, while the bacteria and protozoa (in the form of cysts and oocysts) have a variety of human and nonhuman animal hosts. The agents are transmitted primarily by the fecal-oral route, and as a result the major source of contamination for water is through contact with human and animal fecal pollution.

Much success has been achieved in controlling the more common forms of water-

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borne diseases, and infectious diseases such as cholera and typhoid fever have been virtually eradicated in the developed world, except for sporadic, imported cases. Progress has been due to the adoption of public health measures as well as the implementation of important water treatment techniques, such as flocculation, filtration, disinfection, and sewage treatment. Conventional disinfection of domestic water supplies, accomplished by the addition of chlorine, is highly effective against certain Gram-negative and intestinal bacteria, such as the coliform group. The level of chlorination for the conventional contact time is, however, not adequate to control many enteroviruses and is significantly deficient in controlling cyst- and oocyst-forming pathogenic protozoa that are ubiquitous in domestic water supply raw waters. Although the practice of flocculation and filtration significantly reduces the numbers of protozoa and enteroviruses, these techniques do not always remove microbial contaminants from drinking water. Thus, failure in the performance of these systems may allow outbreaks of waterborne disease.

The potential public health threat posed by waterborne microbial pathogens have attracted renewed attention, both within the scientific community and among the public. Once thought to be under control, they are now referred to as "emerging or reemerging" pathogens. Emerging infectious diseases can be defined as infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range.[4] Agents for which a particular route of transmission is newly recognized and agents (previously unidentifiable) that are now known because of advances in detection methods should also be included in this definition.[5] It is mainly because of advances in epidemiologic and detection methods that about 30 microorganisms have been newly identified as human pathogens during the last 25 years.[6] Not only are many of these responsible for recent waterborne epidemics, but more familiar waterborne pathogens are reemerging to cause significant disease.[7]

Due to the many different infectious agents, reservoirs, and asymptomatic infected individuals, complete eradication of waterborne diseases may not be possible. However, it should be possible to control these agents as long as they can be detected and monitored. Coliforms, the traditional indicators of pathogens, are beginning to fail in some cases by giving misleading information and despite advances being made in microbial testing techniques, some pathogens in drinking water remain undetected. The presence of pathogens in water only becomes evident when a number of people become ill with a waterborne illness and a common source of drinking water is identified. The identification of the specific microorganism that caused the illness may, however, not always be possible. In fact, no agent is identified as the specific cause in 50% of waterborne disease outbreaks. Thus, there is a great need for more appropriate methodologies, both for routine monitoring and for investigating disease outbreaks.

This article provides an overview of the emerging waterborne pathogens, the factors contributing to their emergence or reemergence, and discusses recent developments in the detection of these pathogens.

II. FACTORS AFFECTING THE EMERGENCE AND REEMERGENCE OF WATERBORNE INFECTIOUS DISEASES

In recent years, several "new or emerging" pathogens have arisen as problems in drinking water production and distribution. Therefore, it is important to appreciate the factors that can contribute to the growing problem of waterborne disease and to under-

stand why these infectious agents are particularly troublesome causes of diseases. These issues are addressed here.

A. Changes in Human Demographics and Behavior

An important factor for the emergence of new pathogens is the increasing number of people who are susceptible to infections with specific potential pathogens. These include immunocompromised persons, such as those infected with HIV and patients receiving immunosuppresive therapy for chronic rheumatologic disease, cancer, and solid-organ transplantation, as well as elderly persons whose immune systems are not as active as in healthy young adults.[6,8] As a result of diminished immune responses, these persons are subject to infections that do not occur in healthy adults or, if they do occur, are much less severe in healthy adults. What this means, in effect, is that these persons are at increased risk for morbidity and mortality due to diarrheal diseases.[9] Other groups that may be disproportionately affected by emerging infections include the very young due to their low levels of immunity, persons being cared for in institutional settings, such as the homeless, migrant farm workers, and others of low socioeconomic status.

In many parts of the world, economic conditions are encouraging the mass movement of workers from rural areas to cities. Rural urbanization allows infections arising in isolated rural areas, which may once have remained obscure and localized, to reach larger populations. Once in a city, the newly introduced infection would have the opportunity to spread locally among the population and could also spread further along road and by air transportation.[4] Furthermore, urban population growth in many parts of the world has resulted in a decay of some of the basic sanitation practices, such as waste water disposal and insufficient supplies of potable water. The emergence of slum areas and shanty towns and their attendant sanitation problems have also resulted in conditions under which disease-causing agents may grow and thrive.

B. Breakdown of Public Health Measures

Although classic public health measures have long served to minimize dissemination and human exposure to many pathogens spread by water, the pathogens themselves often still remain, albeit in reduced numbers, in reservoir hosts or in the environment, or in small pockets of infection.[4] Thus, they are often able to take advantage of the opportunity to reemerge if there are breakdowns in preventative measures. For example, the rapid spread of cholera in South America may have been abetted by reductions in chlorine levels used to treat water supplies.[10] Also, the widely publicized U.S. outbreak of waterborne *Cryptosporidium* infection in Milwaukee, Wisconsin, was in part due to a nonfunctioning water filtration plant.[11] Limitations in both surveillance and the availability of appropriate diagnostic tests furthermore constrain public health efforts to prevent and control outbreaks.

C. Microbial Adaptation

Microbes are constantly evolving and may include changes in virulence and toxin production. The most prominent example is pathogenic *Escherichia coli* strains that may have taken up virulence genes by horizontal gene transfer, resulting in very potent new pathogens, the enterohemorrhagic *E. coli* (EHEC).[12] Selection for antibiotic-resistant bacteria and drug-resistant protozoa has also become frequent, driven by the wide and

sometimes inappropriate use of antimicrobial drugs in a variety of applications.[13] Pathogens can acquire new antibiotic-resistance genes from other, often nonpathogenic species, in the environment.[14] Such adaptation often results in "new", more deadly strains against which humans have limited resistance.

D. Changes in Agricultural Practices

The emergence of new pathogens may also be prompted by changes in agricultural production methods. As the number of livestock farms are dwindling, farms are consolidated into intensive farming operations, which means concentration of animal waste, thereby resulting in increased pollution of rivers and streams by agricultural waste and runoff.[15] Because several of the new pathogens of concern in drinking water have known or suspected animal hosts, concern has grown over the potential for direct or indirect animal-to-human transmission through drinking water supplies contaminated with animal wastes containing these pathogens.

III. NEW AND REEMERGING MICROBIAL PATHOGENS OF CONCERN

Estimates indicate that about 90% of the illness associated with domestic water supplies are related to microbial agents, only about 10% are due to chemical agents.[16] The commonly recognized waterborne pathogens consist of several groups of enteric bacteria, viruses, and protozoa. Viruses and protozoa differ from bacterial contaminants in important ways. Because they are environmentally inert, they do not replicate in water and environmental samples. Furthermore, unlike bacterial pathogens, human enteric viruses and protozoal parasites are environmentally stable,[17] are resistant to many of the traditional methods used to control bacterial pathogens,[17] and have notably low infectious doses.[18]

A. Pathogenic Bacteria

A considerable number of newly recognized pathogens from fecal sources (e.g., *Campylobacter*, *E. coli* O157, and *Helicobacter* species) as well as some new pathogens comprising species of environmental bacteria that are able to grow in water distribution systems (e.g., *Yersinia*, *Aeromonas*, and *Mycobacterium* species) are being recognized as increasingly important causes of human disease.

1. Campylobacter

Thermophilic *Campylobacter* species, particularly *C. jejuni* and *C. coli*, are now being recognized as common causes of acute gastroenteritis in most parts of the world.[19] They are typically transmitted by contaminated poultry products, unpasteurized milk, and water. The infective dose of *Campylobacter* cells is low; it has been estimated that approximately 500 cells of *C. jejuni* can cause human illness.[20] Moreover, the presence of *Campylobacter* cells does not correlate with the level of microorganisms that are indicators of fecal contamination in water.[21]

Several studies have been conducted to determine the survival potential of campylobacters in the environment.[22-24] These studies have shown a low survival potential of only a few hours at high temperatures (37°C), but the survival potential increased with decreasing temperatures and reached several weeks at temperatures of 4°C. The survival was enhanced by the presence of other microorganisms and especially in biofilms.[23]

Furthermore, *Campylobacter* spp. are able to exist as viable but nonculturable (VBNC) stages,[24,25] the virulence of which is not clear.[22,26]

Contaminated drinking water has been the cause of several outbreaks of campylobacter enteritis.[27-29] The use of unchlorinated surface water and the secondary contamination of drinking water in storage reservoirs are the main risks for contamination with *Campylobacter* spp. Campylobacters have been found in high numbers (10 to 10^5 cfu/100 ml) in raw sewage[24,30] and they occur in fecally contaminated surface waters ($<10^1$ to 10^2 cfu/100 ml), with high numbers being correlated with contamination from wild birds.[31]

2. Enterohemorrhagic Escherichia coli

Enterohemorrhagic *E. coli* (EHEC) represent a subset of the so-called VTEC (verotoxin-producing *E. coli*) or STEC (Shiga toxin-producing *E. coli*) and was first recognized as a foodborne pathogen in 1982.[32] The route of transmission of EHEC is mostly direct animal contact or the consumption of contaminated beef or dairy products. EHEC causes hemorrhagic colitis with bloody diarrhea, but in children and the elderly it can result in hemolytic-uremic syndrome, often with a fatal outcome.[33] The most prominent representative of the EHEC is *E. coli* O157:H7. From epidemiologic data, it is estimated that the infectious dose of *E. coli* O157:H7 may be less than 100 organisms.[34]

Laboratory experiments have revealed the EHEC are able to grow at temperatures from 8°C to 48°C. Prolonged survival of *E. coli* O157:H7 in water has been reported and a 2-log-unit reduction was observed after 5 weeks at 5°C.[35] Because of methodological problems, EHEC have not been isolated from drinking water supplies, but there is epidemiologic evidence that waterborne infections occur via recreational water, well water, contaminated public water, and paddle pools.[28, 34-39]

3. Helicobacter pylori

H. pylori infection is widespread throughout the world,[40] and it is strongly associated with gastroduodenal disease, including chronic active gastritis, peptic and duodenal ulcer disease, and gastric cancer.[41] The natural history and other aspects of the epidemiology of *H. pylori* infection are still unclear. The mode of transmission of *H. pylori* is also unknown,[42,43] but fecal-oral transmission is highly probable.[44]

When *H. pylori* is exposed to variable environmental conditions, the cells enter a VBNC state and changes in morphology, metabolism, and growth patterns are observed.[45] Laboratory studies[45-47] have demonstrated that *H. pylori* can survive in freshwater and sterile, distilled water (7°C) for 10 and 14 days, respectively. These studies also found evidence that the viable, nonculturable coccoid form of *H. pylori* can survive for up to 10 days at 4°C in tap water. Low levels (10^2 cfu) of the coccoid *H. pylori* forms are needed to induce an acute inflammatory reaction in the stomach mucosa of mice, but higher levels (10^4 to 10^6 cfu) of the vegetative rod form are needed to induce a similar reaction.[48,49]

H. pylori has not yet been isolated from environmental water samples by traditional culture techniques, but its presence in such samples has been shown using techniques such as filtration, immuno-concentration, polymerase chain reaction (PCR), and hybridization with specific probes.[44,50,51] Reports have indicated no significant correlation between the occurrence of total coliforms or *E. coli* in the water and the presence of *H. pylori*.[52]

4. Yersinia enterocolitica

Certain strains of *Y. enterocolitica* (e.g., serotypes O3, O5, O8, and O9) are known to have virulence factors[53,54] and are pathogenic to humans, causing gastrointestinal infections. The routes of infection have not been elucidated completely, but contaminated food and water are the most likely sources. Most of the environmental isolates of *Y. enterocolitica* are nonpathogenic serotypes or so-called atypical *Y. enterocolitica*-like organisms.[55,56] *Y. enterocolitica* is able to grow at very low temperatures (4°C) and may survive for a long time in the environment.[57] Only a few outbreaks of gastrointestinal illness have been reported to be associated with *Y. enterocolitica* in the environment, in sewage, in surface water with fecal influent,[58,59] and in drinking water.[60,61]

5. Aeromonas

Aeromonas species are commonly found in soil and water. Reported densities in water range from 10^2 to 10^3 cfu/ml in river water to 1 to 100 cfu/liter in groundwater. There is some evidence to suggest that a high proportion of environmental isolates may produce enterotoxins,[62,63] and several reports have suggested an association between gastroenteritis and aeromonads in drinking water.[64-66] They have also been found in high numbers in raw sewage (10^6 to 10^8 cfu/ml) and in sewage effluents (10^3 to 10^5 cfu/ml),[67] and these high population densities appear to be related to fecal pollution. Aeromonas have been found to occur in drinking water biofilms and to be protected from disinfection with chlorine within these biofilms.[68]

6. Mycobacterium

The genus *Mycobacterium* comprises both the strictly pathogenic species (e.g., *M. tuberculosis* and *M. leprae*) as well as a wide range of atypical (nontuberculosis) mycobacteria, which may be pathogenic (e.g., *M. avium* and *M. intracellulare*). Atypical mycobacteria are ubiquitous in the aqueous environment and have been isolated from all parts of the drinking water treatment and distribution system.[69,70] Several species among the environmental mycobacteria are opportunistic pathogens and are most commonly associated with pulmonary disease.[71] The incidence of *M. avium* complex infection in HIV-infected patients has dramatically increased, causing further speculation about the possible role of water in the transmission of this agent.[72]

B. Parasitic Protozoa

1. Giardia and Cryptosporidium

Since 1981 enteric protozoa have become the leading cause of waterborne disease outbreaks for which an etiologic agent could be determined.[73] The most important human pathogens are *Giardia lamblia* and *Cryptosporidium parvum* of which the infectious stage is a cyst and oocyst, respectively. In healthy persons, these pathogenic protozoa cause subclinical infections and self-limiting diarrhea. In infants, immunocompromised persons, or those with underlying illnesses, *C. parvum*, especially, can cause very severe, even fatal diarrhea. Although *Giardia* is frequently identified in drinking water, *Cryptosporidium* has become the most important contaminant found in drinking water and evidence indicates that it is the third most common enteric pathogen worldwide.[74] To date, the most prominent recorded public water outbreaks of *Cryptosporidium* have occurred in Carrollton, Georgia, in 1987, involving 13000 symptomatic cases,[75] and in Milwaukee, Wisconsin, in 1993, involving 403,000 symptomatic cases.[11]

The features of these organisms that may facilitate their environmental spread and contamination are the large numbers of cysts and oocysts excreted with the feces of infected hosts and their lack of, or reduced, host specificity.[76,77] The innoculum required to establish infection is very low and humans can be infected with as few as 10 to 100 cysts or oocysts.[18,78,79] Also, the thick-walled cysts and oocysts are very resistant to environmental stress conditions and can survive for weeks and months in the environment.[80,81] The standard levels of chlorine and ozonation used in water purification plants is not effective against *Cryptosporidium* and *Giardia* spp.,[79,82] but optimized filtration processes can reduce the number of cysts and oocysts by approximately 2 to 3 orders of magnitude.[83,84] However, flocculation and filtration do not "kill" or inactivate these agents, but rather remove them from the water to the sludge.

Contaminated recreational water (e.g., lakes, rivers, or swimming pools) frequently have been associated with waterborne outbreaks of giardiasis[85,86] and cryptosporidiosis.[87-90] Both *G. lamblia* and *C. parvum* have also been recognized as a public health threat in treated drinking waters. Between 2 and 7700 cysts or oocysts/100 liters of water have been found in implicated drinking water supplies.[78] Surveys of raw water supplies indicated that the occurrence of cysts and oocysts are widespread,[90-92] and they are often found in a high percentage of surface waters,[93,94] especially when these are contaminated by sewage or manure. The occurrence and concentration of the organisms in surface waters are likely to be higher in developing countries in which contamination of water by human and animal waste is more common.[78]

2. Microsporidia and Cyclospora

Microsporidia and *Cyclospora* represent groups of protistan organisms that are attracting much attention as emerging pathogens. The microsporidia are very small obligate intracellular parasites of vertebrates and invertebrates.[95] Potentially waterborne,[96] they are recognized as causing disease primarily in immunocompromised persons.[97] Very little is known about the occurrence and distribution of human pathogenic microsporidia in the environment. Because of its small size, microsporidia may survive filtration, and studies thus far indicate that the pathogen will be fairly resistant to many drinking water disinfectants.[98] *Cyclospora cayetanensis* was identified as a new protozoan pathogen of humans in 1993.[99] *Cyclospora* routes of transmission are still unknown, although the fecal-oral route, either directly or via water, is thought to be highly probable.[100-102] Recently, *Cyclospora cayetanensis* has been associated with the consumption of contaminated water,[77,79,100,103] but the magnitude of cyclosporiasis as a form of community-acquired diarrhea is unknown.[104]

C. Viruses

1. Enteric Viruses

Human enteric viruses are recognized as important causes of waterborne illness. In addition to causing gastroenteritis, enteric viral infections can also result in meningitis, respiratory disease, and encephalitis. The failure to report outbreaks of mild gastrointestinal disease as well as inadequate diagnostic technology, which has limited the detection of many enteric viruses in environmental samples, has resulted in a drastic underestimate of the true scope and importance of waterborne viral infection.[105] In recent years, it has become clear that many different viruses can be transmitted via drinking water. More than a 100 enteric viruses, all of which are pathogenic to man, have been reported.[106] Recently identified pathogens include the

enteric adenoviruses, calicivirus, astrovirus, and the Norwalk family of agents.

Volunteer studies have revealed that some enteric viruses are highly infective (i.e., one or a few tissue culture infectious units suffice to initiate an infection), with the risk of infection being 10- to 10,000-fold higher than that for bacteria at the same level of exposure.[107] Enteric viruses are not only excreted in high numbers in the feces of infected individuals, but many of them tend to be persistent in the aquatic environment and are able to survive in water in an infectious state for months. Their inactivation and/or removal by water treatment processes varies by virus type and treatment conditions.[108,109] Several enteric viruses (e.g., Norwalk virus and rotaviruses) and enteroviruses are reported to be relatively chlorine resistant,[110] and viruses have been isolated from drinking water that meets bacteriological standards,[106] that contains the recommended level of free chlorine,[110,111] and in drinking water biofilms.[112]

Frequently, fecally contaminated water has been identified as a source of viral infections. Cultivable enteric viruses have been detected in surface waters, ground waters, and treated drinking waters in concentrations ranging from 647 pfu/liter[113] to 1 pfu/1000 liters.[108] Rotaviruses, some adenoviruses (serotypes 40 and 41), and hepatitis E virus are frequently associated with waterborne disease outbreaks in developing areas in Africa, Asia, and Mexico. These outbreaks have been linked to fecally contaminated water and inadequate chlorination.[73,114] Norwalk and related small, round structured viruses (such as Snow Mountain agent, Hawaii agent, Sapporo agent) are the leading cause of epidemic viral gastroenteritis in older children and adults in the United States. Numerous outbreaks linked to drinking water, recreational water, ice, and environmental contamination have been documented.[115-119]

2. Other Fecally Derived Viruses

Several other viruses are recognized enteric pathogens or putative pathogens. These include the group B rotaviruses, which were first reported in connection with a waterborne outbreak in China in 1984,[120] and pestiviruses, which were identified in stool specimens from children under 2 years of age with diarrhea.[121] Viruses known to be etiologic agents of diarrhea in animals have also been observed in the feces of persons with gastroenteritis. These include coronaviruses,[122] toroviruses,[123] and picobirnaviruses.[124,125] There are, however, no reports of waterborne transmission of enteric pestivirus, coronavirus, torovirus, or picobirnavirus. Methods to detect them in water have not been developed, and there is no information about the survival of these viruses in water.[126]

V. DETECTION OF ENTERIC MICROBIAL PATHOGENS IN WATER

Generally, indicator organisms are used to establish the potential presence of fecal contamination in raw and drinking water. Not necessarily pathogenic themselves, fecal coliforms, total coliforms, *E. coli*, enterococci, and bacteriophages are all examples of organisms that when present are viewed as predictive of the potential presence of enteric pathogens, because they have the same fecal source as the pathogenic organisms. Tests for coliform bacteria are standardized and relatively easy and inexpensive to use.[127,128] They are therefore more rapidly administered than tests determining the presence of individual pathogenic microorganisms in water. Despite being successful in predicting possible health risks in many circumstances, the presence of coliforms in a particular water sample does not necessarily correlate well with the inci-

dence of disease, and they tend to be poor models for enteric protozoa and viruses.[73] Thus, there is a need to examine newer approaches to monitoring the microbiological quality of water that will lead to a reduction of waterborne disease transmission.

A number of problems are encountered in determining the presence of microbial pathogens in water. For each group of microbes, whether protozoa, viruses or bacteria, the method must cope with a different set of conditions or characteristics that can complicate the task of identifying particular microbes. Because the concentrations of enteric organisms are low in water, their detection in water typically starts with some type of concentration process such as filtration. This is followed by a process to recover the pathogen from the filter and then by methods to assay and characterize the pathogenic microbes.

A. Enteric Bacteria

1. Traditional Detection Techniques

Bacteria can be recovered and concentrated from water by a variety of filtration methods. The most widely used filtration method for recovering bacteria is membrane filtration using microporous membranes typically composed of cellulose esters.[128] Following filtration, the cells recovered on the membrane filter can be directly assayed or after culturing.

Various approaches for culturing the target bacteria following filtration are generally followed. The target bacteria can either be cultured by preenrichment and enrichment methods using broth media or the filter can be placed on differential and selective media to allow the development of discrete colonies of the target pathogens. Alternatively, the bacteria can be washed off the filter surface and reconcentrated in a small volume of suspension medium, which is plated on agar media. Quantitative results are preferably obtained by colony counts on the surface of agar media, with or without the presence of the filter used for concentration.[128] Often, organisms may be present in water samples but are unculturable. The bacteria are still viable (exhibit low levels of metabolic activity), but they fail to develop colonies on most traditional solid culture medium. Such a viable but nonculturable state (VBNC) state has been described for many pathogenic bacteria including *H. pylori*, *Campylobacter*, *E. coli*, *Vibrio cholerae*, *Vibrio* spp., and *Legionella pneumophila*.[25,45,129-132] Also, nutrient limitations and environmental stressors, such as disinfectants used during water treatment, can produce unpredictable physiological and morphological changes in many waterborne bacterial pathogens. This makes their isolation and identification problematic and specialized handling procedures are required for their resuscitation.[133] By making use of nonselective or less selective media, and other, less stressful culture conditions, the number of culturable cells in a population of VBNC, injured, or stressed bacteria may be increased. To confirm their identity, the cultured bacteria or bacterial colonies can be characterized by making use of a variety of methods, such as subculturing on other differential and selective media, biochemical, metabolic and other phenotype analyses, immunological analyses, and nucleic acid-based analyses.

Enzyme immunoassays (EIA), which relies on the use of enzymatically or fluorescently labeled monoclonal antibodies specific for individual groups and species of bacterial pathogens, have been used widely to detect and quantify pathogenic bacteria in water. Many different EIA methods are available for both quantitative and qualitative analysis.[23,134,135] However, the antibodies may exhibit cross-reactions that compromise the specificity of the test or they may fail if the

original protein target, such as a cell surface protein, undergoes changes under environmental conditions.[136] Furthermore, no information regarding the viability of the organisms are obtained.[137] However, by combining microscopic examination with chemical treatments for enzymatic activity, the viability of the concentrated and purified bacterial pathogens may be assayed. For example, an approach combining fluorescent antibody and tetrazolium dye reduction, which measures dehydrogenase activity, has been used to successfully enumerate viable *E. coli* O157:H7 in water.[138]

2. Nucleic Acid-Based Detection Methods

The discovery of a large number of bacterial toxins and other virulence factors has led to powerful methods, such as gene probes and PCR, for detecting and identifying pathogenic bacteria in water, as well as bacterial pathogens in a VBNC state that may not be detected by culture-dependent techniques. Two general types of gene probes that have been developed in recent years are DNA probes complementary to a single gene or a small region of a gene[139-142] and DNA probes complementary to genus- or species-specific regions of 16S rRNA[143-145] for use in whole cell *in situ* hybridization (FISH).[146-148] In contrast to the use of DNA probes complementary to genes, FISH allows for greater sensitivity, and it is possible to detect a single bacterial cell without first amplifying the bacterial population by growth. There are, however, limitations to using FISH for the detection of waterborne bacterial pathogens as stressed or starved bacteria are less reactive, bacterial debris in water may interfere with fluorescence, and inanimate material may bind to probes.

PCR amplification-based methods, being more rapid and very sensitive, have become the most used molecular approach for detecting infectious bacterial agents in water.[149-151] The major limitations to using PCR for detection is the presence of substances inhibitory to PCR[152] and the possibility of amplification of nonviable cells.[153] Inhibition phenomena have made bacterial DNA purification an important preliminary step for the PCR reaction. Subsequently, numerous techniques for separating and purifying the DNA have been described, resulting in varying purification levels of the DNA.[154,155] In recent years, the use of immunomagnetic beads attached to specific antibodies have become a popular approach in facilitating the capture, concentration, and purification of target bacteria prior to DNA extraction.[156]

Several assays based on the direct detection of indicator and pathogenic bacterial cells in environmental water samples by filtration and PCR[157-160] have been developed. The use of combinations of methods such as immunomagnetic capturing of strains, PCR, and detection of immobilized amplified nucleic acids by hybridization have been proven to be useful for the detection of waterborne pathogenic bacteria that are difficult to culture from environmental sources, for example, *Y. enterocolitica*,[161,162] and *H. pylori*.[50,163] A disadvantage of the above approaches is that they may detect dead as well as viable bacteria. An indirect approach is thus usually adopted for assaying the viability of bacteria from water samples by first culturing bacteria prior to PCR detection.[164-166] Not only does an enrichment procedure dilute any sample-related inhibitors present, but dead bacteria are diluted as well, thus reducing the probability of detecting them by the subsequent PCR assay. Thus, it becomes possible to relate detection to pathogen infectivity. In addition, allowing pathogens to multiply amplifies target nucleic acids, thereby facilitating their detection. Despite these advantages, this approach still relies on culturing and so it cannot be applied to current noncultivable pathogens.

B. Enteric Parasitic Protozoa

1. Traditional Detection Techniques

Parasitic protozoa are usually present in low concentrations in contaminated water and therefore must be concentrated from large volumes of water before detection. *Giardia* cysts and *Cryptosporidium* oocysts are concentrated by filtration through yarn-wound filters after which retained particulates are eluted from the filters and reconcentrated by centrifugation. The pelleted cysts and oocysts are then separated from particulate debris by flotation on Percoll-sucrose gradients followed by subsequent detection.[167] While recovery efficiencies as high as 100% have been reported,[168] recovery is generally poor and greatly affected by water quality and particulate matter.[168,169] Alternative methods such as calcium carbonate precipitation of particulates can concentrate *Cryptosporidium* oocysts from water with concentration efficiencies as high as 68%.[170]

Traditional detection methods for parasitic agents from water sample concentrates have been based on immunofluorescent staining of filtered sample concentrates.[167] The sample concentrate is filtered through cellulose acetate filters, and commercial kits that use fluorescein isothiocyante-labeled monoclonal antibodies are applied for immunofluorescent staining. The stained filters are examined under an ultraviolet microscope, and cysts and oocysts are classified according to immunofluorescence, size, shape, and internal morphologic characteristics. Despite having been used successfully in surveys of raw[93] and finished[94] waters, the method is extremely limited as it is time-consuming and expensive, requires highly skilled and experienced personnel, and does not indicate viability of the detected cysts or oocysts. In addition, cross-reactions of monoclonal antibodies with algal cells and debris can interfere with the interpretation of results.[171] Commercially available enzyme-linked immunosorbent assays (ELISA) are becoming available for the parasitic protozoa and have the promise to be highly sensitive and rapid.[172] While presently applicable only to direct use on fecal samples, these ELISA systems may become suitable for use with environmental samples, particularly if the samples are concentrated prior to analysis.

Detection methods based on cell sorting or particle counting have been developed for the separation, purification and detection of *Giardia* and *Cryptosporidium*. The Fluorescence-Activated Cell Sorting (FACS) system is a laser-based particle counter that is able to simultaneously sort particles, sense fluorescence, and determine size.[170,173,174] Because fluorescent antibodies are used in the FACS procedure and cross-reactions with other fluorescent objects can occur, it is recommended that sorted fluorescence objects of the right size and shape be confirmed on an epifluorescence microscope. Because the FACS processing is rapid and eliminates most of the contaminating debris and background fluorescence, microscopic observation of the sample is less fatiguing. However, general application of this technology is limited by the high cost of the instruments and the requirement of a skilled, dedicated analyst. Furthermore, infectious and noninfectious organisms cannot readily be distinguished.[167]

Membrane exclusion/permeability fluorogenic dyes such as propidium iodide[175,176] and 4',6-diamidino-2-phenylindole (DAPI)[177] have been used to assess the viability of protozoan cysts and oocysts. Detection of these differentially stained cysts and oocysts has greatly been facilitated by microscopic and analytical imaging methods such as charge-coupled device (CCD) cameras[173,178] and confocal laser scanning microscopy.[179] More recently, viability assays based on nucleic acid staining of *C. parvum* oocysts with SYTO-9, SYTO-59, and hexidium have

been developed and used in conjunction with immunofluorescent staining methods for the detection of *Cryptosporidium* oocysts.[180,181]

The infectivity of the spores of some of the important human microsporidia and oocysts of *C. parvum* can be assessed by culturing on susceptible mammalian host cells where spores germinate or oocysts excyst and active stages of the organisms can proliferate in the cells.[182,183] The living stages can subsequently be detected (e.g., after immunofluorescent or other staining) and quantified by counting the numbers of discrete living stages of groups (foci) of them. Detection is also possible by PCR, immunoblotting, and electron microscopy. However, for waterborne protozoa such as *G. lamblia* and *Cyclospora cayetanensis* culture from the cysts or oocysts recovered in a water sample is still not possible.

2. Nucleic Acid-Based Detection Methods

Molecular approaches based on DNA hybridization have been developed for the detection of *Giardia*[184] and *Cryptosporidium.*[185] These methods are generally regarded as inadequate due to high detection limits and large sample volumes that are impractical for most hybridization protocols without further pathogen concentration. The development of amplification methods such as the PCR has greatly facilitated the direct detection of low levels of pathogens in environmental samples. Consequently, several PCR methods for the detection of *Giardia* and *Cryptosporidium* in water have been developed[186-190] as well as a multiplex PCR for the simultaneous detection of these protozoa in environmental water samples.[191] Methods that combine genus-specific PCR with restriction fragment length polymorphism (RFLP) have been applied to detect and speciate *Cryptosporidium* oocysts and have thus provided a means whereby pathogenic and nonpathogenic species can be distinguished.[192,193]

While PCR methods have the potential to detect a single infectious unit and may be applied to discriminate pathogenic from nonpathogenic species, they have remained limited in their application because of enzymatic inhibition, the absence of quantitative assays, and the inability to discriminate between viable and nonviable organisms. Several approaches have been adopted to address these issues. In order to overcome inhibition of the PCR, methods combining immunomagnetic separation with PCR amplification have been developed to detect low levels of *Giardia* cysts[194] and *C. parvum* oocysts.[193, 195-197] In addition, PCR methods for quantifying the number of *C. parvum* oocysts in water are being developed. Two recent quantitative PCR methods allowed the quantification of 30 to 50 oocysts seeded into municipal water samples[198] and 258 oocysts/2 liter of sludge,[199] respectively.

Determining the viability of waterborne protozoa remains a technical challenge. Early approaches in determining the viability of waterborne *C. parvum* oocysts relied on an excystation protocol before DNA extraction to allow differentiation between viable and nonviable *C. parvum* oocysts by detection based on PCR.[200,201] More recently, various mRNAs have been evaluated in reverse transcriptase (RT)-PCR assays as potential markers for cyst and oocyst viability.[189] RT-PCR assays directed at mRNA encoding the enzyme amyloglucosidase (CPAG),[202] t-tubulin[203] and the heatshock protein (hsp70)[204] have been successful in distinguishing viable from nonviable oocysts. Integrated approaches that combine immunomagnetic separation for the recovery of *C. parvum* oocysts with *in vitro* cell culturing followed by PCR (CC-PCR)[205] or RT-PCR[206] have demonstrated that the recovered oocysts retained their infectivity.

C. Enteric Viruses

1. Traditional Detection Techniques

The low level of viruses found in environmental waters necessitates the concentration of virus by adsorption to a microporous filter.[207] The adsorbed viruses are subsequently eluted by passing a small volume of beef extract, as eluent, through the filter. Alternative techniques that further concentrate and purify intact virions from the sample concentrate by physicochemical methods include polyethylene glycol (PEG) precipitation, Sephadex gel chromatography, chelation of multivalent cation impurities, and ultrafiltration.[126,208] However, care should be taken in applying these treatments because injury or damage of the target viruses during these processes can interfere with their subsequent detection by cultivation or other methods.

Traditional methods to directly detect viruses in water after concentration have been based on the ability of enteric viruses to infect live mammalian cells in culture. Various cell types have been used, but the sensitivity of each cell line to different viruses must be taken into account as some are highly selective for certain virus types.[209-211] Primary or secondary human embryonic and primate kidney cell cultures appear to be the most sensitive host systems for enteric virus isolations.[106] Assays of viruses in cell cultures can be quantified using quantal (most probable number or 50% endpoint) or enumerative (plaque) methods. The plaque assay is generally more precise and accurate than the quantal assay because relatively large numbers of individual infectious units can be counted directly as discrete, localized areas of infection (plaques). However, conventional culture assays have been limited because some of the most important waterborne enteric viruses do not replicate (e.g., Norwalk and related viruses and some caliciviruses) or replicate poorly (e.g., rotaviruses and hepatitis A virus) in cell culture.[17] These viruses are not detected in water unless alternative methods such as ELISA assays or nucleic acid-based detection methods are applied directly to concentrated samples.

2. Nucleic Acid-Based Detection Techniques

Nucleic acid-based detection methods, especially *in vitro* enzymatic amplification of specific viral genomic nucleic acid sequences by PCR and RT-PCR (for the detection of RNA viruses) followed by detection of the amplified nucleic acid by hybridization have emerged as sensitive and specific approaches for the detection of many enteric viruses.[212-214] PCR amplification methods have since advanced to the point where they have been applied successfully to investigating waterborne outbreaks caused by nonculturable human caliciviruses and in surveying drinking water and environmental sources for the presence of enteroviruses, Norwalk virus, rotavirus, and adenoviruses.[208,215-220]

Despite enormous strides in the ability to detect human enteric viruses with PCR, the technique is still limited by the absence of effective concentration methods, the presence of enzymatic inhibitors, and the inability to distinguish between infectious and noninfectious virions. Several extraction techniques have been developed to isolate and purify viral nucleic acid from sample concentrates.[221,222] However, these techniques expose labile viral nucleic acid, often single-stranded RNA, to conditions that could lead to degradation before analysis, thereby causing a loss of target and a decrease in sensitivity. In addition, the infectivity of the viruses cannot be determined. One approach to the nucleic acid-based detection of intact and hence potentially infectious enterovi-

ruses is virus capture mediated by antibody-antigen complexes. This approach has been used to isolate, concentrate, and purify virus particles from environmental water samples for subsequent nucleic acid amplification and detection.[223-225] The use of an integrated cell culture-RT-PCR procedure followed by nested PCR, for enhanced sensitivity, have been used to detect infectious human astrovirus, enterovirus, and adenovirus (types 40 and 41) in surface water samples, in recreational marine water samples and in sewage.[226-228]

VI. SUMMARY AND CONCLUSIONS

Various demographic and other changes combined with complacency about the role of infectious diseases in general have brought about a resurgence in waterborne infectious diseases, and with it challenges that are unprecedented in recent times. A World Health Organization report[3] states that about 80% of all diseases and over one-third of deaths in developing countries are the result of people consuming contaminated water. Not only are the diseases debilitating, but they also consume valuable time, with about one-tenth of each person's productive time sacrificed to water-related diseases. While waterborne diseases may be considered to be problems of underdeveloped countries with inadequate sanitary practices, there is increasing recognition that industrialized, developed countries also have significant public health problems caused by use of untreated, partially treated, or inadequately treated domestic water supplies. The detection and identification of microbial contaminants in drinking water supplies therefore should, be viewed as a high priority.

Many years of experience have shown that monitoring microbiological water quality by standard indicator systems is and has been an important means for the supply of safe drinking water. Indicator methods are simple and easy to use, cheap, and cover a wide range of pathogens. However, current indicators leave little room for improvement. These methods have served relatively well but are less accurate and informative than what is needed. The main limitations are lack of specificity and slow response. Furthermore, current indicators are poor for addressing parasites and viruses. The recognition of new waterborne pathogens as well as reemerging ones posing increased risks due to newly acquired virulence properties and other traits requires examination of newer approaches to monitoring the microbiological quality of water if control and prevention of waterborne diseases are to be realized.

Recent developments, particularly in molecular biological research in the last few years, have resulted in a wide range of new methods, principally based on the detection of nucleic acid material and its amplification. They offer a novel, more sensitive and specific way of detecting microorganisms. They can also identify organisms that would not be detected with current culture techniques and can be used to track new pathogenic entities, including variants of otherwise harmless microorganisms. There are probably very few groups of microorganisms that have not been located with these amplification techniques and several test kits have already been commercialized. Despite the success of these prototype alternative and rapid methods in detecting human enteric microbial pathogens in water, multiple barriers must be overcome before they can be used to routinely assess water quality and the microbiological safety of drinking water.

The relationship between detection by molecular approaches and the subsequent viability or infectivity of waterborne enteric pathogens remains a concern. Despite using various approaches in order to address this issue, the combination of nucleic acid methods with culture methods appears to be the

most promising approach for sensitive and specific detection and quantification of infectious waterborne pathogens. However, this approach is still not ideal because it cannot be applied to the noncultivable human enteric pathogens, and it is expensive, time-consuming, and not readily amenable to routine diagnostic work. In addition, methods used for purifying and concentrating the target microbes and their nucleic acids from water, so that they are free of contaminants that may interfere with the analysis, need further improvement, consolidation, and simplification.

Evaluation studies and standardization of the procedures are also necessary to force the approval of new methods by health authorities, this being the basic requirement for routine application. Further research is thus needed to develop and refine the prototype protocols into collaboratively tested methods that could be routinely and expeditiously used to evaluate the microbiological safety of water. Furthermore, issues of cost, personnel efficiency, and equipment needs must be evaluated to ensure that the challenge of diagnosing these emerging infections will be met. Ultimately, the true measure of the value of rapid molecular methodologies is whether they will represent improvements over conventional methodologies in terms of achieving a reduction of waterborne disease and an improvement of human health.

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