Section VI

Analysis of zoonotic microorganisms

A. Dufour and D. Till

Management of water quality has been applied mainly to receiving waters contaminated by point sources of human pollution. Waters affected by point sources of pollution are usually subject to regulation, because the pollution frequently impacts bathing beaches or shellfish harvesting waters, as well as drinking-water supplies. Water resources contaminated by dispersed, unidentified sources of pollution, the type usually associated with animals, have not been given special attention until fairly recently. This is the result of a greater awareness of emerging waterborne pathogenic zoonotic microorganisms and improved technical methods to measure water quality. This, in turn, has led to some technical problems that were not anticipated when microbial indicators of faecal contamination were first proposed as a means of monitoring water quality. These problems include the methodology and microbes traditionally

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used to monitor water quality and the recognition of emerging zoonotic pathogens as a waterborne risk, resulting, in some instances, from changing host population behaviour patterns.

At the beginning of the 20th century, Escherich noted that harmless, easily cultivated bacteria occurred in faeces. The organism was suggested for use as an indicator of the presence of faeces from warm-blooded animals in water. The practice of using *Bacillus coli* (later named *Escherichia coli*) as a measure of water quality was adopted, and, over the years, it was used in many forms, dependent on the practical methodology available. Coliforms, faecal coliforms, and *E. coli* were measured, respectively, over time as new specific methods became available. The measurement of *E. coli* in water served as evidence of the presence of faeces and even as a guide to how much faecal contamination was present. This approach appeared to work, because it was applied to waters near relatively small urban centres with poor infrastructure, little water treatment, and populations with little mobility. However, this approach to measuring water quality has been shown to have many inadequacies in identifying public health risk, no doubt related to extending its practical use to unintended applications that have never been validated.

Urban centres have expanded beyond their central confines, to the extent that suburban spread is encroaching on feral animal habitat. Another pressure not envisioned when microbial indicators, such as *E. coli*, were proposed is the commercialization of aquatic food resources harvested from waters that were frequently contaminated with human and animal faecal wastes. Similarly, the practice of confining large numbers of animals to feedlots has created a situation where very large volumes of faecal waste frequently reach waterways, leading to pollution of aquatic food sources and water resources used for recreation. In addition, much of the increased leisure time available to humans is spent on recreational water activities far from urban centres and nearer to natural animal habitats.

This population movement has led to regulations attempting to govern the safety of all surface waters, including those with non-anthropogenic sources of faecal contamination. However, the means by which we monitor surface waters to provide evidence that they are free of faeces — and, therefore, zoonotic enteric pathogens — has changed little in the last 100 years. The microbial indicator of faecal contamination has not changed, and the methodology for measuring these faecal indicator organisms has changed very little. The methods still rely on bacterial growth in culture media to enumerate their presence in water samples. This usually requires a 24-h period of growth before results are obtained, presenting a situation where the potential risk associated with a water body is detected long after a risk activity has occurred.

The risk of illness associated with exposure to foods harvested from animal-contaminated waters or the risk of illness due to direct exposure to these waters is real, but largely unquantified. Although we are fully aware of waterborne zoonotic illnesses through individual cases and outbreaks of illness, we have no way of predicting illness associated with animal-contaminated waters. Our current system for monitoring water quality is designed to protect human health from human-derived pathogens. Unfortunately, the source of faecal indicator bacteria used to monitor water quality is not specific to humans. Currently used indicator methodology cannot distinguish an *E. coli* from animals from an *E. coli* whose source is human. This shortcoming has led many countries to treat animal-contaminated water as if it were water contaminated by humans and therefore of equal risk.

Some solutions to these problems are presented in the chapters of this section. The means to define risk associated with waterborne zoonoses, techniques for identifying sources of animal pollution, and techniques for measuring the safety of animal-contaminated water are discussed with respect to providing tools and information for managing water resources.

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Managing risk of waterborne zoonotic disease through water quality surveillance

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21.1 WHAT IS THE RISK?

The extent of the worldwide disease burden resulting from potential or actual waterborne zoonoses is a growing concern. Until recently, the focus of concern for water-related illness and associated research have been directed towards contamination by human effluent. Microbiological water quality guidelines for recreational waters often included advice that exposure to animal faecal

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microorganisms is a lesser risk than exposure to pathogens of human origin. Guidelines for drinking-water concentrated on catchment protection and on the effectiveness of chlorine and other treatments for eliminating risk from human viruses. Accordingly, there have been very few studies carried out on the effect of animal faeces as a source of waterborne zoonotic infections.

Studies show that there is a swimming-associated pollution-related illness risk (WHO 2003), although results are equivocal for studies that have included findings for beaches impacted by animal faecal material. In a Hong Kong study (Cheung 1988; Holmes 1989), illness rates for two marine beaches impacted by animal (pig) wastes were lower than those for seven other beaches (although total swimming-associated illness rates were still statistically different from zero). In a Connecticut, USA, freshwater study (Calderon *et al.* 1991), no association of gastrointestinal risk with densities of faecal indicator bacteria was reported. Those results have been questioned, in that the data could be reinterpreted to imply that the faecally related health risks were very similar to those found in studies on waters impacted by human effluent (McBride 1993). In a New Zealand study of seven populous marine beaches, no substantial differences in illness risks were found between the human and animal waste-impacted beaches, but both were markedly different from the control beaches (McBride *et al.* 1998).

21.2 EMERGING PATHOGENS

In recent years, several so-called "new" or "emerging" pathogens of animal origin have presented as potential sources of waterborne infection. Few of these pathogens are of recent origin, and some may have been causing illness for a long time, but they were not previously identified due to a lack of suitable isolation and identification methods. These include *Campylobacter jejuni*, *Yersinia enterocolitica*, *Giardia lamblia*, and *Cryptosporidium parvum.*

In addition, there is the emergence of bacteria with new virulence factors, resulting in very potent new pathogens. One example is the pathogenic *Escherichia coli* strains, the enterohaemorrhagic *E. coli* (EHEC) suspected of taking up virulence genes from *Shigella* by horizontal gene transfer.

Members of this so-called new or emerging pathogens group all have an animal reservoir from which they are transmitted to humans, directly or via the environment. In contrast to the classical zoonoses, such as salmonellosis, these emerging zoonoses may not cause apparent clinical conditions in infected animals. Thus, apparently healthy animals can excrete to the environment disease-causing microorganisms of public health significance. Several of these pathogens are resistant to environmental stress (*Cryptosporidium*) and have a

low infective dose (EHEC). Even low-level contamination of water environments may lead to infections and illness in the exposed population.

Surface runoff and point source pollution from pastoral agriculture may introduce pathogenic microorganisms such as *E. coli* (EHEC), *Campylobacter*, *Cryptosporidium*, and *Giardia* into streams and rivers (Geldreich 1996), compromising their suitability for contact recreation and as a source of drinking-water. Small, unprotected rural drinking-water supplies may be a particular concern.

To perform a risk analysis for zoonotic pathogens in recreational water and drinking-water sources, it is necessary to understand the ecology of these organisms and their risk as infective agents. Results from epidemiological studies, together with ecological data, are the basis for effective resource protection and risk assessment for both recreational water use and drinking-water supplies. There are significant gaps in both epidemiological and ecological data available for effective risk assessment. What is known is that the increased incidence of waterborne infections such as campylobacteriosis and the challenge to traditional water treatment systems posed by cysts and oocysts of protozoa such as *Giardia* and *Cryptosporidia* present a situation whereby there is a heightened awareness of the disease risk from waterborne pathogens of zoonotic origin.

21.3 ANIMAL VERSUS HUMAN FAECAL CONTAMINATION

An inherent part of public health water quality surveillance is the evaluation of microbiological results from a water monitoring programme designed to identify unusual trends or patterns and episodes of human and animal faecal material entering waterways used as source waters for drinking-water, for recreational activities, and as a source of shellfish.

An essential element in the surveillance programme is the reporting of water monitoring results to regulatory agencies responsible for informing concerned parties of actual or potential health risk. The target audience for surveillance information will typically include public health officials at local and national levels, water suppliers, local administrators, communities, and water users.

Recreational waters, shellfish harvesting waters, and waters used as a source of drinking-water may be subject to faecal contamination from point sources (usually human domestic wastes) and from non-point sources (highly dispersed animal excreta). Public health authorities maintain the quality of waters for users either by intervention (e.g., requiring treatment) or by limiting access to water sources (e.g., protected catchments). Another approach is to regulate the quality

of the waters by limiting the level of faecal material that can encroach on these resources. Regulating water quality is the most widely used approach to protect public health. Monitoring the water using methods that quantify the density of indicator bacteria in water samples is common practice to ensure water safety. Faecal material and potential enteric pathogens that may be associated with faeces are limited by statute in recreational, shellfish harvesting, and drinking-water source waters to minimize illness in consumers of drinking-water or shellfish products and users of recreational water. There is no agreement on how these waters are monitored. In most shellfish harvesting water jurisdictions, water quality is measured using total coliforms or faecal coliforms. Recreational waters are monitored using *E. coli*, enterococci, faecal coliforms, faecal streptococci, or total coliforms. Sources of water that will be used for drinking-water are monitored in some jurisdictions using faecal coliforms, total coliforms, or *E. coli.* The actual indicators used in different environments are described in numerous diverse international and national guidelines. The World Health Organization is making serious efforts to harmonize approaches to monitoring recreational and other waters.

Water quality monitoring systems that use different indicator bacteria do have some things in common. The water quality limits for drinking-water sources and shellfish harvesting waters are not risk based — i.e., they are not based on a water quality–health relationship. Limits for recreational waters in many countries are based on a relationship between water quality and swimming-associated gastrointestinal disease. A second common feature is that the indicator bacteria cannot distinguish between faeces produced by humans and faeces discharged by animals. This is a significant shortcoming of currently used indicator bacteria. It is intuitive to believe that non-human faeces probably carry fewer pathogens that might be hazardous to humans. For example, viruses that are specific to humans do not normally occur in animals; therefore, the risk from animal faeces has been considered not to be equivalent to that associated with human faeces.

The dilemma is that the presence of such faecal indicators may or may not be an indication of actual risk from pathogens at that time and are of little use in determining if their faecal source is human or animal. Only detailed knowledge of the sources of faecal material in the catchment impacting on a waterway, be they human or animal in origin, and data as to the spatial and temporal loadings of expected pathogens from such sources can assist the assessment of a public health risk.

21.4 LIMITATIONS OF PATHOGEN DETECTION METHODS

The most straightforward approach to assessing public health risk is to monitor microbial pathogens directly. Although effective assays for many pathogens exist, currently these are often expensive and technically complicated. Pathogens may be rare, difficult to culture, and irregularly distributed, yet highly infectious, even at low doses. Because of this rare and uneven distribution, an enrichment step for culture-based detection or some type of stacked polymerase chain reaction (PCR), such as nested PCR for molecular detection, is often required. In most cases, this extra step makes accurate quantification difficult, potentially introducing culture or PCR bias. In addition, detection by PCR does not yield information about the viability/infectivity of the detected organism, as the target of detection is a gene sequence, not a viable pathogen.

The most important limitation to direct detection of pathogens is the number of assays for different pathogens that are required. Furthermore, faeces from both humans and animals may contain as-yet-unidentified pathogens or pathogens for which no assays exist. Newer technology promises the ability to assay for perhaps thousands of indicators and pathogens with a single gene chip or microarray. Microarrays consist of probe sequences (up to many thousands) immobilized in separate locations on a support such as a gel matrix bound to a glass slide. Either DNA or RNA recovered from the environment serves as the target for hybridization to the array of probes. Microbial ecologists have pioneered this approach for monitoring natural populations (e.g., see Guschin *et al*. 1997; Rudi *et al*. 2000; Liu *et al*. 2001; Small *et al*. 2001; El Fantroussi *et al*. 2003). They are working on overcoming the technology's current problems, such as non-specific hybridization (Guo *et al*. 1994; Mir and Southern 1999; Peplies *et al*. 2003). Straub and Chandler (2003) have discussed applications of microarray technology to the detection of multiple pathogens in water.

The availability of such advances will in the future provide a means to further evaluate the relationship of indicators to pathogens and enhance knowledge of pathogen loadings from human and animal sources. However, at present, such resources are limited in availability, very expensive, and, in many instances, still in development.

21.5 LIMITATIONS TO USE OF FAECAL BACTERIAL INDICATORS

Because of limits to direct monitoring of pathogens, it is standard practice to monitor easy-to-culture faecal indicators such as *Escherichia coli* and faecal

enterococci. Recently, molecular detection of indicator organisms has also come into use; for example, a rapid detection method for faecal pollution based on real-time quantitative PCR of enterococci has been proposed (Santo Domingo *et al*. 2003). Epidemiological studies have led to human health standards based on exposure to these indicators in drinking-water, recreational water, and shellfish waters (e.g., see Cabelli *et al*. 1982; Cabelli 1983; Dufour 1984; Cheung *et al.* 1990; McBride *et al.* 1998).

Limitations to the use of bacterial indicators have become apparent. For example, both *E. coli* and enterococci can survive and proliferate outside of animal intestines, in tropical soils and vegetation and temperate habitats such as freshwater algal wrack around the Great Lakes (Hardina and Fujioka 1991; Hagler *et al*. 1993; Anderson *et al*. 1997; Fujioka *et al*. 1999; Solo-Gabriele *et al*. 2000; Desmarais *et al*. 2002; Whitman *et al*. 2003). This calls into question their reliability as indicators in these habitats.

In addition, viral and protozoan pathogens are not well correlated with standard bacterial indicators (for some recent examples, see Wyer *et al*. 1995; Barrett *et al*. 2001; Jiang *et al*. 2001; Noble and Fuhrman 2001; Lemarchand and Lebaron 2003). The processes that control the survival and removal of microbes in water, such as competition, ultraviolet radiation, temperature, predation, and transport (e.g., see Rozen and Belkin 2001), differ among viruses and eukaryotes, and even among different types of bacteria. Thus, monitoring bacterial indicator species may not be sufficient to assess human health risk.

A number of investigators have suggested that the presence of indicator viruses is a better predictor of contamination by faecal viruses than is the presence of indicator bacteria. Commonly used viral indicators include coliphages (Funderburg and Sorber 1985; Havelaar *et al.* 1986; Borrego *et al.* 1987; Havelaar *et al.* 1990; Palmateer *et al.* 1991; Araujo *et al.* 1997; Paul *et al.* 1997) and bacteriophages of *Bacteroides fragilis* (Tartera and Jofre 1987; Tartera *et al.* 1989; Puig *et al.* 1999; Araujo *et al.* 2001). These have traditionally been assayed by plating on susceptible cell cultures, but there are now sensitive molecular assays for a large number of human viruses, including enteroviruses and adenoviruses (reviewed in Griffin 2003). There is still a lack of epidemiological data linking occurrence of viral indicators with pathogens; a lack of information on differential survival of viral groups, especially RNA versus DNA viruses; and a lack of knowledge about population/geographic distribution of viruses in faeces.

Since microbial indicators first came into use, microbial ecologists have amassed substantial information about bacteria in natural and artificial populations. The phrase “the great plate count anomaly” (Staley and Konopka 1985) was coined to describe the very large difference between the number of microbes counted by direct microscopic examination of a particular sample and

the number that can be grown on agar plates. Typically, culturable cells represent only a fraction of a per cent of total cells present. Furthermore, cultured strains do not adequately represent the diversity of types in the habitat. This is true even in well studied copiotrophic habitats, such as the lower intestines of humans and animals. Molecular surveys of bacterial diversity in faeces and the gut have revealed largely unknown strains (Wilson and Blitchington 1996; Pryde *et al.* 1999; Suau *et al.* 1999; Bernhard and Field 2000; Daly *et al.* 2001; Favier *et al.* 2002; Holben *et al.* 2002; Hold *et al.* 2002; Leser *et al.* 2002; Zoetendal *et al.* 2002). It has been known for some time that the community composition of microbial cells in a sample changes drastically when the sample is held in a container and incubated (Ferguson *et al.* 1984; Fuchs *et al.* 2000), even when the container is very large and incubation takes place under natural conditions. It cannot be assumed, therefore, that the indicator types present in a culture proportionally represent what was present in the original habitat or sample.

To avoid problems of culturability and culture bias, investigators typically profile microbial communities by a combination of PCR and sequencing (e.g., see Rappé and Giovannoni, in press). However, because PCRs from mixed templates such as microbial communities are often biased (Suzuki and Giovannoni 1996; Suzuki *et al.* 1998), this approach does not result in a proportional representation of the community. PCR-based surveys must be considered to be qualitative, not quantitative, unless specialized techniques such as real-time quantitative PCR are used and shown to be quantitative.

21.6 SUMMARY AND CONCLUSIONS

The increasing presence of zoonotic pathogens in surface waters provides new and unique challenges for water resource managers. Zoonoses, such as leptospirosis, *E. coli* O157 gastroenteritis, and cryptosporidiosis, have been well defined, and their animal sources have been linked to human disease caused by exposure to contaminated water. Other emerging zoonoses that may be transmitted by faeces through the waterborne route have not been as well defined. The presence of animal faeces in water is currently not identified, because traditional bacterial indicators of faecal contamination cannot distinguish human faeces from animal faeces. Because of their inability to distinguish human from animal faecal contamination, resource managers and regulators have elected to treat all faecal contamination as equally hazardous to human health. This approach frequently results in the closure of beaches and shellfish harvesting areas that are affected by stormwater runoff that carries faecal indicator bacteria of non-human origin. The risk of exposure to these waters contaminated by animals is unknown. Studies that have attempted to

define the risk associated with swimming in animal-contaminated water have not given a clear indication that there is an excess illness rate related to this type of exposure. These equivocal results do not lead to the conclusion that all faecally contaminated waters should be treated alike. New research to define the risk posed by animal faecal wastes to users of water resources and indicator systems that identify animal contamination of surface waters are needed. The availability of more research data that would meet the latter two information needs would significantly improve our ability to manage water resources.

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22

Faecal source identification

K.G. Field[1]

22.1 INTRODUCTION

When there is faecal contamination in water, it is necessary to determine the source, both to mitigate the contamination and to estimate human health risk, as the source will determine which pathogens are present. Both human and animal faecal contamination pose a threat to human health. For example, human faeces are commonly associated with the spread of *Salmonella typhi*, *Shigella* spp., hepatitis A virus, and Norwalk-group viruses; animal faeces spread many diseases, including *Salmonella*, *Escherichia coli* O157, *Giardia*, *Cryptosporidium*,

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and hepatitis E virus. Even technologically advanced parts of the world like North America have experienced recent severe waterborne disease outbreaks (e.g., *Cryptosporidium* in Milwaukee, Ohio, USA, in 1993; *E. coli* O157 in Walkerton, Ontario, Canada, in 2000). The human population may be exposed via drinking-water (Alary and Nadeau 1990; MacKenzie *et al*. 1994; Preston *et al*. 2000), swimming or bathing (Cabelli *et al*. 1982; Keene *et al*. 1994), irrigation, food preparation, and ingestion of contaminated shellfish (McDonnell *et al*. 1997; Fayer and Lewis 1999; Berg *et al*. 2000).

A variety of warm-blooded, and even some cold-blooded, animals contain faecal indicators in their faeces (e.g., see Harwood *et al*. 1999). Thus, the presence of indicator species in water is not sufficient to determine the source of faecal contamination.

Diagnosing the sources of faecal contamination in water is often called bacterial source tracking or microbial source tracking. Since naturally occurring microbes are ubiquitous in water, with bacteria occurring at an average density of 10^6 cells/ml in natural waters, and because the real concern is faecal pathogens in water, not microbes or bacteria, a preferable term is faecal source identification.

22.2 CHEMICAL METHODS OF FAECAL SOURCE IDENTIFICATION

Substances such as caffeine, faecal sterols and stanols, laundry brighteners, surfactants, fragrances, pesticides, and polycyclic aromatic hydrocarbons can be used to detect human and non-human faecal contamination and to determine urban sources of faecal contamination (Nichols *et al*. 1993; Leeming *et al*. 1996; Edwards *et al*. 1998; Sinton *et al*. 1998; Burkhardt *et al*. 1999; Standley *et al*. 2000; Isobe *et al*. 2002; Puglisi *et al*. 2003; Suprihatin *et al*. 2003). Standley and colleagues (2000) compared several of these so-called "molecular tracers" and concluded that a combined index of caffeine and fragrance levels was an effective identifier for human sewage; a ratio between particular steroids made an effective identifier for agricultural input; and a different steroid ratio identified wildlife input. Similarly, profiling of seven sterols in South Australian metropolitan catchments suggested areas of human, dog, and bird faecal impact (Suprihatin *et al*. 2003).

Although chemical indicators and molecular tracers can identify recent faecal inputs, their spread, transport, and persistence in water are unlikely to be correlated with that of pathogens. Currently, there are no epidemiological data that correlate these markers with human risk. In addition, some chemical indicators, such as laundry brighteners and caffeine, are culturally dependent and might not be effective outside developed areas.

22.3 MICROBIAL METHODS OF FAECAL SOURCE IDENTIFICATION

Microbial methods for faecal source identification can be divided into library-dependent and library-independent approaches. In this context, a "library" is a set of bacterial isolates from faecal samples, tested using the method of source discrimination; it is also called a "host origin database." Source identification occurs by a statistical comparison between patterns from the library and environmental isolates. Library-dependent methods include both phenotypic and genotypic tests.

Library-based approaches are labour-intensive, requiring extensive sampling both to prepare the library and to test environmental isolates. All library-based methods have complex requirements for adequate sample size, representativeness, and geographic stability (Wiggins *et al*. 2003; Harwood *et al*., in press; Myoda *et al*., in press). Limited data on geographic stability suggest that for most methods, libraries are not cosmopolitan, and thus a separate library for each locale or watershed may be required (e.g., see Souza *et al*. 1999; Hartel *et al*. 2002).

The average rate of correct classification (ARCC) is a statistical estimate of the ability of a library to correctly classify isolates pulled from the library. ARCCs reported in some studies have been quite high (e.g., see Dombek *et al*. 2000; Guan *et al*. 2002). However, the size of the library influences its ARCC. Small libraries have higher ARCCs than large libraries, but small libraries are not as representative and are therefore not as good at classifying novel isolates (from outside the library) as are large libraries (Wiggins *et al*. 2003). Thus, the ARCC is not a good estimator of the ability of the method to identify faecal sources and can be misleading. Because most methods of faecal source tracking have been assessed only by calculating ARCCs or by application in field studies, where the "real" answer is not known, the ability to compare the effectiveness of the methods is limited.

Microbial methods for faecal source tracking can also be divided into culture-dependent and culture-independent methods. Culture-dependent approaches require growing microbes from water samples. The microbes may be viruses or (more commonly) faecal indicator bacteria, such as *E. coli* or enterococci. The particular source identification method may require selective growth conditions, so that growth itself is diagnostic. Alternatively, a diagnostic test may be applied to the microbial isolates. All library-dependent methods are also culture-dependent.

22.3.1 Library-dependent, culture-dependent methods

22.3.1.1 Phenotypic methods: MAR and CUP

In the multiple antibiotic resistance (MAR) method, also called antibiotic resistance analysis, isolates of *E. coli* or enterococci are tested against panels of antibiotics in order to discriminate among human and various animal sources of faecal pollution; for some examples, see Parveen *et al*. (1997), Hagedorn *et al*. (1999), Wiggins *et al*. (1999), and Harwood *et al*. (2000). Antibiotic resistance is often borne on plasmids, is under strong selection, and is influenced by antibiotic exposure of the host population. Thus, it is not a geographically stable trait; libraries of strains from known sources must be constructed for each new geographic region being tested.

However, the MAR method is inexpensive and low-tech, making it readily available to a broad variety of investigators. In addition, its usefulness has been "proven," as it has been successfully applied to identify and mitigate faecal pollution originating from cattle (Hagedorn *et al*. 2003).

Carbon-source utilization (CUP) has recently been applied to faecal source identification (Hagedorn *et al.* 2003; Wallis and Taylor 2003; Harwood *et al*., in press). In CUP, faecal bacterial isolates are tested against a variety of carbon sources using a commercial kit. This method gave poor identification of sources and a high rate of false positives in a study in which it was applied to blind samples from known sources (Griffith *et al*., in press; Harwood *et al*., in press).

Several investigators have suggested serotyping indicator organism isolates to distinguish faecal sources (reviewed in Scott *et al*. 2002). Parveen and colleagues (2001) achieved a relatively good separation between human and non-human faecal isolates (in a library) with this method, but concluded that its expense made it more appropriate for application in combination with another method.

22.3.1.2 Genotypic methods

Ribotyping, repetitive extragenic palindromic polymerase chain reaction (REP-PCR), amplified fragment length polymorphism (AFLP), and pulsed-field gel electrophoresis (PFGE) are fingerprinting techniques producing bar code-like patterns for each bacterial isolate. In a recent comparative study that analysed samples of known composition, accuracy of faecal source identification by these methods was influenced by whether the investigators simply scored similar patterns as related or required that patterns matched exactly in order to identify them; the latter approach led to a higher percentage of correct source identifications (Myoda *et al.*, in press).

Ribotyping (e.g., see Samadpour and Chechowitz 1995; Parveen *et al*. 1999; Carson *et al*. 2001; Hartel *et al*. 2002; Myoda *et al*., in press) involves growing indicator bacterial isolates, extracting DNA, amplifying 16S rRNA genes, digesting amplification products, and detecting patterns of restriction fragments. This approach was popularized by extensive commercial application in the USA, and currently a number of studies using it are under way. The process is available in an automated format, although most investigators do it manually.

Several different versions of REP-PCR have been tested for faecal source identification, differing in choice of primers (e.g., see Dombek *et al.* 2000; Carson *et al.* 2003; McLellan *et al.* 2003). In this method, DNAs extracted from faecal indicator isolates are amplified with PCR primers complementary to conserved repetitive elements in bacterial genomes. The result is patterns of different-sized fragments, depending on the location of the conserved elements.

In AFLP, genomic DNA from faecal indicator isolates is digested with restriction enzymes, linkers are ligated to the restriction fragments, and fragments are amplified with PCR primers that target the linkers, generating fingerprints. Guan and colleagues (2002) recently compared this method with antibiotic resistance patterns and 16S rDNA sequencing with the same set of isolates and concluded that the AFLP approach had a relatively good ARCC, considering that their samples covered a broad geographic area; however, the method was expensive.

PFGE generates a bacterial fingerprint by digesting the entire genome with rare-cutting restriction enzymes and separating the resulting large fragments with a specialized gel apparatus made to separate large fragments of DNA. The equipment required is expensive and the gel separation step is slow, but the resulting fingerprints are the "gold standard" in another field, epidemiological studies of foodborne pathogens. This method is just beginning to be applied in source identification studies (e.g., see Myoda *et al.*, in press). McLellan and colleagues (2003) make the point that the fine level of genetic discrimination achieved by PFGE may not be necessary to discriminate source-specific clones.

The underlying assumption of these genotypic methods is that host-specific genetic structure exists across indicator bacteria populations (McLellan *et al.* 2003). In a comparative study using multiple REP-PCR assays and PFGE, McLellan and colleagues (2003) found multiple closely related subclusters of genotypes, which were mostly host-specific. However, the relationships calculated for members of the clusters varied depending on the primers or techniques used. Because of very high levels of diversity, both REP-PCR and PFGE would require a separate library for each geographic area.

22.3.2 Library-independent, culture-dependent methods

22.3.2.1 Viral methods

Phage of *Bacteroides fragilis* can distinguish human and animal faecal pollution, as certain strains of *B. fragilis* will grow bacteriophages only from human sewage and others will support phage growth from both human and animal faeces (Puig *et al.* 1999). Similarly, two serotypes of F+ RNA coliphages, Types II and III, are found in association with human faecal contamination, whereas Type IV is found in association with animal faecal contamination and Type I occurs in both human and animal faeces (Hartly *et al.* 1975; Furuse *et al.* 1981). Growth of these coliphages in an appropriate cell culture, followed by serotyping, identifies human and non-human faecal contamination in water. Recently, serotyping has largely been replaced by molecular typing (Hsu *et al.* 1995).

These viral methods are limited to discriminating between human and animal sources. Little is known about differential survival of the various types, which would affect the ability to discriminate over time. In addition, the markers appear to be irregularly distributed in populations and may work better in some geographic areas and when faecal sources comprise multiple individuals (such as from sewage) rather than single individuals (Noble *et al.*, in press).

22.3.3 Advantages and limitations of culture-based methods

Culturing faecal indicator organisms is relatively inexpensive and low-tech, making it broadly available. However, this advantage is lost if the source identification method that is applied to the cultured isolates is high-tech and expensive (e.g., PFGE and AFLP). Another advantage of culturing is that it provides an enrichment step, increasing the numbers of target microorganisms and providing single strains in isolation. Finally, culture-based methods often use standard public health indicators such as *E. coli* or enterococci, for which some information about survival and transport is available.

Disadvantages are that these methods are limited to testing easily cultured microbes. Many pathogens, and even the most common faecal bacteria, may be very difficult to grow. In addition, the composition of microbial communities changes drastically when cultured (e.g., see Ferguson *et al.* 1984). This “culture bias” has rarely been considered in faecal source identification. Phenotypic traits such as antibiotic resistance may be under strong selection, which would influence relative survival. Genotypic traits (e.g., ribotypes) are markers for clonal types, which may have phenotypic differences that affect their relative survival in culture.

If the culturing step did accurately reproduce the proportions of indicator organisms present in the original population, a significant advantage of culture-based approaches would be that they would allow ready quantification of different sources in the population. It should be possible to experimentally confirm or disprove this proportional survival, but this has not been attempted.

It is likely that some of the traits used for source discrimination in culture-based methods could be directly assayed from water samples without an intervening culture step. This would require much more specific tests (perhaps highly specific PCR primers) than are currently used for culture-based methods.

22.3.4 Culture-independent methods

In these methods, some marker is assayed directly from a water sample or from DNA extracted from a water sample, without an intervening culture step. Many of these methods assay specific genes by PCR; this approach is sometimes called "host-specific PCR."

22.3.4.1 Viral methods

A number of viruses can be monitored directly in water, without culturing (reviewed in Griffin *et al.* 2003). The presence of human or bovine viruses indicates the presence of human or cattle faecal pollution. Examples include human adenoviruses (Pina *et al.* 1998; Jiang *et al.* 2001), human enteroviruses (Tsai *et al.* 1993; Griffin *et al.* 1999; Noble and Fuhrman 2001), and bovine enteroviruses (Ley *et al.* 2002). Monitoring for viruses typically requires very large water samples; concentration of such large samples frequently concentrates PCR-inhibitory substances as well, which may interfere with detection (Jiang *et al.* 2001; Straub and Chandler 2003). To increase sensitivity, investigators may use nested PCR, which makes it difficult or impossible to detect quantitatively (Jiang *et al.* 2001). A real-time quantitative PCR assay has been used successfully to quantify enteric viruses (Monpoeho *et al.* 2000; Boehm *et al.* 2003). The viral methods are effective in detecting human sewage, although they may not detect faeces from individuals or small groups of humans (Noble *et al.*, in press).

22.3.4.2 Bacteroidetes faecal markers

Species in the genus *Bacteroides* have host-specific distributions that could make them useful as faecal source identifiers (Allsop and Stickler 1985; Fiksdal *et al.* 1985; Kreader 1995). However, as this group is anaerobic, it was not practical to use them for this purpose until the advent of routine molecular detection. *Bacteroides* and related genera (class Bacteroidetes) comprise a large

proportion of the faecal flora in warm-blooded animals, making them a relatively easy target for detection. In addition, they are genetically diverse, are limited to animal body cavities, are unlikely to survive long after release into receiving waters, and show species- or group-specific host distributions. Bernhard and Field (2000a, 2000b) used terminal restriction fragment length polymorphisms (T-RFLP) and length heterogeneity PCR (LH-PCR) to identify human and ruminant-specific Bacteroidetes sequences, which were used to design PCR primers to amplify human or ruminant markers out of faecally contaminated waters. Subsequently, clone library analysis and subtractive hybridization were used to design primers for other faecal sources (L.K. Dick, M.T. Simonich, S.P. Walters, and K.G. Field, unpublished data). These primers sensitively and specifically detect faecal pollution in small water samples and distinguish its source (Field *et al.* 2002; Bernhard *et al.* 2003). Primers are based on sequences present in faecal samples, not on sequences from isolated strains, taking advantage of dominant microbial constituents of faeces that have never been grown (e.g., see Wilson and Blitchington 1996; Suau *et al.* 1999; Bernhard and Field 2000b; Leser *et al.* 2002). Analysis of faecal samples from several locations in the USA and elsewhere suggests that the primers are widely geographically applicable (L.K. Dick, S.P. Walters, and K.G. Field, unpublished data). Dick and colleagues developed a quantitative PCR assay for the Bacteroidetes group and showed that Bacteroidetes dosage in sewage was well correlated with *E. coli*, opening up the possibility of using molecular detection of Bacteroidetes as a rapid quantitative indicator of faecal pollution (L.K. Dick and K.G. Field, unpublished data).

22.3.4.3 Toxin genes from E. coli

A heat-stable enterotoxin from enterotoxigenic *E. coli*, the STIb toxin, is associated with human faecal waste; its gene is targeted by PCR primers to detect human faecal pollution (Oshiro and Olson 1997). Similarly, the STII toxin gene is associated with pig faeces and is targeted by primers that detect pig faecal contamination (Khatib *et al.*, in press). The heat-labile enterotoxin, LTIIa, is associated with cattle faecal waste; its gene is the target of PCR primers to detect faecal pollution from cattle (Khatib *et al.* 2002). These markers are generally specific (with occasional exceptions; Field *et al.*, in press), are temporally and geographically stable, and occur at a high enough prevalence for culture-independent detection as long as a large enough number of cells is screened (Oshiro and Olson 1997; Khatib *et al.* 2002). They may also be detected following a culture step in which *E. coli* is enriched (Field *et al.*, in press). Because of the sporadic distribution of enterotoxigenic *E. coli*, this approach may not detect individual faecal samples (Field *et al.*, in press).

22.3.4.4Bifidobacterium

Like the Bacteroidetes group, several groups have suggested using *Bifidobacterium* species to identify the source of faecal pollution (Resnick and Levin 1981; Carillo *et al.* 1985; Sinton *et al.* 1998; Rhodes and Kator 1999; Bernhard and Field 2000a; Nebra *et al.* 2003). Resnick and Levin (1981) found that members of the genus *Bifidobacterium* could not be cultured after 5 h in fresh water or 10 h in salt water. Carillo and colleagues (1985) also observed very low survival of *B. adolescentic* in a tropical environment and suggested that the genus could be used to detect only very recent faecal contamination. Bernhard and Field (2000a) developed human- and ruminant-specific T-RFLP and LH-PCR markers from uncultured *Bifidobacterium* in faeces. However, the *Bifidobacterium* markers were difficult to detect out of faecally polluted natural waters, suggesting that survival of this group after release is insufficient for this application (Bernhard and Field 2000a). Nebra and co-workers (2003) developed a probe that detects 16S rDNAs from *B. dentium*, a species thought to be limited to humans, and another probe intended to detect a number of animal-specific *Bifidobacterium* species. Following amplification of *Bifidobacterium* 16S rDNAs using general primers, they used their probes to differentiate human and animal samples. Although the *B. dentium* probe could detect most human sewage samples and a few individual human faecal samples, with a low number of false positives, the animal probe was unsuccessful. The amplification/probing approach does not lend itself to quantification. In addition, basing primers solely on published sequences from isolated strains may miss the most common *Bifidobacterium* strains in faeces, thus limiting the genetic variability that could be used for primer/probe design.

22.3.4.5 Community sampling

Animal species may have faecal bacterial communities that differ enough to allow them to be distinguished from one another by community fingerprinting methods (e.g., see Cho and Kim 2000; Satokari *et al.* 2001; Simpson *et al.* 2002; Tannock 2002; Zoetendal *et al.* 2002; Nagashima *et al.* 2003). In a comparison of small-subunit rDNA community fingerprints generated by T-RFLP, LaMontagne and colleagues (2003) differentiated cattle and dog faeces and sewage. Application of the method in a comparative study with samples of known composition resulted in low identification of sources with high false positives, however (Griffith *et al.*, in press). Experimental evidence suggests that PCR from mixed templates may be biased (e.g., see Suzuki and Giovannoni 1996; Suzuki *et al.* 1998; Becker *et al.* 2000). For this method to work for diagnosing sources of faecal pollution in water, PCR bias must be minimized and the PCR must be quantitative.

22.3.5 Advantages and limitations of culture-independent methods

Culture-independent methods have the advantage of sampling the entire population present in the sample, with no culture bias. In addition, they are simpler and quicker than culture-based methods; they may require only a few hours to detect faecal pollution and identify its source. They do not require prior preparation of a "library," as markers are in most cases universal. They are not limited to easy-to-culture microbes, but may instead use difficult-to-grow but common faecal microbes or mine the uncultured genetic diversity in faeces for source-specific markers.

A drawback of using any markers other than standard public health indicators is that their survival and correlation with standard faecal indicators and pathogens are poorly known. Since regulations are based on public health indicator bacteria, any other markers must be correlated with public health bacteria in order for managers to use them. Epidemiological studies that predict health outcomes are also needed.

A further limitation of the culture-independent methods is that markers for only a few animal species are currently available; wildlife species especially are not represented. Most of the culture-independent methods result in presence/absence data on marker occurrence; quantitative assays are needed. Finally, for any of these markers, it is important to test their geographic range and temporal variability.

A relatively small variety of gene sequences has been tested for use in host-specific PCR for faecal source identification. It is likely that many other appropriate targets will be identified. With primers or probes targeting multiple targets to identify each faecal source, perhaps combined in a microarray format with primers or probes for pathogens, faecal source identification could be rapid and reliable.

22.4 A COMPARATIVE STUDY OF FAECAL SOURCE IDENTIFICATION METHODS: THE SCCWRP STUDY

It is difficult to compare faecal source identification methods, as most have been applied only to analyse real-life samples from watersheds, where the "real" answer is not known. The Southern California Coastal Water Research Project (SCCWRP) and the US Environmental Protection Agency (EPA) sponsored a comparative source identification study (Griffith *et al.*, in press). Study participants were asked to identify the faecal source(s) in water samples containing human, cattle, dog, or gull faeces, sewage, or a mixture. Along with

replicate unknown water samples, participants were supplied with samples of the faeces used to create the unknowns. Study participants used many of the methods reviewed above, including coliphage and virus-based approaches (Noble *et al.*, in press), phenotypic culture-based approaches (MAR and CUP) (Harwood *et al.*, in press), genotypic culture-based approaches (ribotyping, REP-PCR, and PFGE) (Myoda *et al.*, in press), and non-culture-based approaches, including *E. coli* toxin genes, community sampling by T-RFLP, and several methods based on Bacteroidetes bacteria (Field *et al.*, in press). Methods were assessed according to their ability to identify whether samples contained or did not contain human faeces, identify each faecal source, and handle freshwater and saltwater samples and samples with humic acids.

Host-specific PCR (of *E. coli* toxin genes or Bacteroidetes markers) was very accurate at identifying samples with human faeces or sewage with no false positives. Several of the other methods, including the phenotypic methods and genotypic library-based methods, identified most or all samples with human input, but did have false positives. The virus-based methods worked well at identifying samples with sewage but less well at identifying samples with human faeces. None of the methods correctly identified all the sources in every sample. The host-specific PCR methods (of either *E. coli* toxin genes or Bacteroidetes markers) were relatively accurate at identifying the species for which they had markers, but did not have markers for all species. Most of the other methods had significant numbers of false positives, although some culture-based genotypic methods (ribotyping, PFGE) performed better than others.

This is the first comparative study using blind samples of known composition to compare faecal source identification methods. Although, due to limitations of the study, some methods did not perform optimally, several broad conclusions could be reached. First, the same approach did not perform equally well in the hands of different investigators, underlining the need for standardization. Second, the rate of false positives for culture-based, library-dependent methods was often higher than had been anticipated based on published ARCCs, underlining the weakness of this statistic. Third, each method had strengths and weaknesses, and no method performed perfectly. Some methods accurately identified human faecal contamination and would be useful when the principal research question is the identification of human input. Some methods were relatively rapid and accurate for some sources, but did not identify all the sources; these would be useful where the principal research objective is to identify the major sources of faecal contamination, to allow rapid mitigation. Other methods were more time-consuming and less accurate, but identified all sources; these would be appropriate where it was important to know all sources, perhaps for health risk assessment. No method was able to accurately quantify the sources.

An important finding of this study came out of the experimental design rather than source identification results. When making up unknown samples, the proportion of each type of faeces in the samples was quantified by estimating the concentrations of *E. coli* in the input human, dog, cattle, and gull faeces. However, since several source identification methods in the study were based on enterococci, not *E. coli*, enterococci concentrations were estimated as well. The relative proportions of the different types of faeces in samples estimated by the two methods were in many cases radically different (Table 22.1).

Table 22.1. Relative proportions of faeces of selected unknown samples from the SCCWRP faecal source identification study, as estimated by counts of *E. coli* and enterococci (unpublished data used with permission of John Griffith and Steve Weisburg, SCCWRP)

Sample	Faecal proportions according to counts of *E. coli*		Faecal proportions according to counts of enterococci	
C	Human 96%	Gull 4%	Human 18%	Gull 82%
E	Dog 86%	Cattle 14%	Dog 54%	Cattle 46%
F	Human 58%	Gull 42%	Human 1%	Gull 99%
H	Cattle 90%	Gull 10%	Cattle 32%	Gull 68%
N	Sewage 58%	Dog 42%	Sewage 4%	Dog 96%
T	Human 44%	Gull 56%	Human 4%	Gull 96%

The very large differences in apparent faecal contributions in identical samples, measured with *E. coli* or enterococci, illustrates a crucial shortcoming of using indicators to assess the risk of pathogen contamination. Unless the correlation of each pathogen to each indicator has been established, indicators cannot be used quantitatively to estimate risk.

22.5 AN IMPORTANT LIMITATION OF FAECAL SOURCE IDENTIFICATION METHODS

Little is known about proportional survival of different microbial groups or markers in water, yet this is basic to faecal source identification. For example, an older method of faecal source tracking, comparing the ratio of faecal coliforms to faecal streptococci, has been largely abandoned, because the two groups survive differently, causing the ratio to change over time (Pourcher *et al.* 1991; Sinton *et al.* 1993). The relative survival of faecal microbes or markers being used for source discrimination has not been investigated; this is a major shortcoming of all source tracking technologies, not just library-based methods. Before source identification can be used to help assess the risk of exposure to pathogens due to faecal contamination of water, researchers must measure the

survival of proposed source-specific markers, in relation to each other, in relation to standard faecal indicators such as *E. coli*, and in relation to pathogens.

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23

Rapid methods for the detection and enumeration of microorganisms in water

D.Y.C. Fung

23.1 INTRODUCTION

The application of rapid methods for water microbiology requires, first and foremost, effective and efficient sampling methods and techniques. Since the number of microorganisms in water is usually low, concentration of the microbes is an important aspect of water microbiology.

The most commonly used method is filtration. Usually, a 100-ml volume of water is filtered through a 0.45-µm-porosity, 47-mm-diameter nitrocellulose membrane and plated on a medium that is selective for specific organisms, such

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as total coliforms, faecal coliforms, and other pathogens. For turbid waters, the labour-intensive multiple-tube, most probable number (MPN) methods may have to be used. For protozoa and viruses, much larger volumes may be used. Elution and dissolution of filter matrix are required; other methods include desorption, elution, acid precipitation, and polyethylene glycol precipitation. Positively and negatively charged filters are also used to trap viruses. The most common method for the enumeration of microorganisms is the total viable cell count method, which uses selective and non-selective agars for growing live bacteria, yeasts, and moulds. This involves sample dilution, application of samples onto petri dishes, pouring melted agar, incubation of solidified agar samples for a specified time and temperature, and enumeration of colony forming units (CFU/ml), depending on the agar used and the colour, shape, size, and fluorescence characteristics of the microorganisms.

This chapter describes some efficient and rapid methods to ascertain total viable cell numbers in water as well as application of genetic and biosensor techniques to detect target pathogens in water for the protection of public health nationally and internationally.

23.2 MODIFICATIONS TO CONVENTIONAL SYSTEMS

In the past 20 years, several modifications of the conventional viable cell count method have been made. The spiral plating method is an automated system to obtain viable cell count. By use of a stylus, this instrument can spread a liquid sample on the surface of a pre-poured agar plate (selective or non-selective) in a spiral shape (the Archimedes spiral) with a concentration gradient starting from the centre and decreasing as the spiral progresses outward on the rotating plate. The volume of the liquid deposited at any segment of the agar plate is known. After the liquid containing microorganisms is spread, the agar plate is incubated overnight at an appropriate temperature for the colonies to develop. The colonies appearing along the spiral pathway can be counted either manually or electronically. The time for plating a sample is only several seconds compared with minutes as in the conventional method (Spiral Biotech 2003).

An ingenious hydrophobic membrane system was also developed with a square bacteriological filter printed with hydrophobic grids to form 1600 squares for each filter. A water sample can pass through the filter assisted by vacuum with the microbes trapped on the filter into the squares. The filter is then placed on pre-poured non-selective or selective agar and then incubated for a specific time and temperature. Since growing microbial colonies cannot migrate over the hydrophobic material, all colonies are localized into a square shape. The analyst can then count the squares as individual colonies. Since there is a chance that more than one bacterium is trapped in one square, the system

has an MPN conversion table to provide statistically accurate viable cell counts (Neogen, Inc. 2003).

A very popular self-contained film system has been developed and widely used internationally. In this system, appropriate rehydratable nutrients are embedded in a series of films in the unit. The unit is a little larger than a credit card. To obtain viable cell counts, the protective top layer is lifted and 1 ml of liquid sample is introduced to the centre of the unit; the cover is then replaced. A plastic template is placed on the cover to make a round mould. The rehydrated medium will support the growth of microorganisms after suitable incubation time and temperature. The colonies are directly counted in the unit (3M Company 2003).

Another convenient system consists of tubes of sterile nutrient with a pectin gel in the tube but no conventional agar. This liquid system is ready for use, and no heat is needed to "melt" the system, since there is no agar in the liquid. After an analyst mixes 1 ml of liquid sample with the liquid in the tube, the resultant contents are poured into a special petri dish coated with calcium. The pectin and calcium will react and form a gel, which will solidify in about 20 min. The plate is then incubated at an appropriate temperature for the proper time, and the colonies are counted the same way as for the conventional standard plate count method (3M Company 2003).

The four methods described above have been in use for almost 20 years. Chain and Fung (1991) made a comprehensive evaluation of all four methods against the conventional viable cell count method on seven different foods, 20 samples each, and found that the alternative systems and the conventional method were highly comparable at an agreement of $r = 0.95$. In the same study, they also found that the alternative systems cost less than the conventional system for making viable cell counts.

A relatively new method, called "Fung's Double Tube Method," has been developed to rapidly enumerate anaerobes such as *Clostridium perfringens* (Fung and Lee 1981). In this method, a larger tube is used to house a water sample (e.g., 1 ml), and then an appropriate amount of agar, such as SFP agar, is introduced into the larger tube. A smaller tube is then inserted into the large tube, thereby creating a thin agar layer between the two tubes. Colonies then formed can be easily enumerated in the system. Since *C. perfringens* grows very quickly at 42 °C, a *C. perfringens* count can be made in about 6 h from polluted environmental water samples. Hawaii is the only US state that utilizes *C. perfringens* as an indicator of faecal contamination of recreational water. Recently, during a research project in Hawaii, a filter system was introduced into the double tubes, and now almost any volume of water can be tested in the double tubes as long as the water (10 ml, 100 ml, or other volume) can be filtered through a membrane.

Several "real-time" instruments for viable cell counts have been introduced. The direct epifluorescent filter technique (Tortorello 1999) involves using vital dye (acridine orange) to stain live bacteria for obtaining viable cell counts in about 1 h. A new system filters cells on a membrane, then lets the cells grow for a few hours; the filter is then stained with vital dyes. Viable cell counts can be made by scanning the colonies by laser (Chemunex Corporation 2003). Another rapid system has been developed that utilizes bacterial ATP to report the growth of microcolonies trapped in a filter in a few hours (Millipore Corporation 2003).

Development of a miniaturized MPN method has been successful in recent years based on the original work of Fung and Kraft (1969). Irwin *et al*. (2000) developed a modified Gauss-Newton algorithm and a 96-well microtechnique for calculating the MPN using spreadsheets. This technique is less cumbersome than many traditional MPN procedures. Similarly, Walser (2000) developed a microtitre plate technology for the automation of microbiological testing of drinking-water. The system was validated for the analysis of aerobic mesophilic counts in drinking-water. The system can cope with low or high bacterial loads, ranging from 0 to 4.3 log CFU/ml. The system reduces the tedium and personnel influence of routine microbiological work and is applicable to many other determinations, such as faecal indicator and other microorganisms in water.

23.3 NEW MICROBIOLOGICAL TESTING TECHNIQUES

Many advances have been made in rapid methods and automation in microbiology in the past 20 years. Fung (2002) comprehensively reviewed the subject in the inaugural issue of the journal *Comprehensive Review in Food Science and Food Safety*, published by the Institute of Food Technologists. The history and key developments in the field were described. In addition, advances in sample preparation and treatments, total viable cell count methodologies, miniaturization and diagnostic kits, immunological testing, instrumentation and biomass measurements, genetic testing, and biosensors were described. Finally, US and world market and testing trends and predictions of future developments were included. Of particular interest are the developments in genetic testing and biosensors, which can be used in water microbiology. Traditional microbiological methods rely on growth characteristics of organisms at various temperatures, colony morphology in various cultural media, biochemical reactions in various carbohydrates, and immunological antibody–antigen reactions. These characteristics are influenced by environmental factors and growth conditions of cells.

23.3.1 Genetic methods: Polymerase chain reaction (PCR)

Genotypic characteristics of a cell are far more stable than phenotypic expressions. Genetic methods have been developing rapidly in the past two decades. First, DNA and RNA hybridization techniques were perfected and widely used to rapidly detect organisms such as *Salmonella*, *Listeria*, *Campylobacter*, etc. More recently, PCR has been widely used for the detection of a great variety of microorganisms in clinical, food, industrial, and water microbiology (Mrozinski *et al*. 1998). Basically, a DNA molecule (double helix) of a target pathogen (e.g., *Salmonella*) is first denatured at about 95 °C to form single strands, then the temperature is lowered to about 55 °C for two primers (small oligonucleotides specific for *Salmonella*) to anneal to specific regions of the single-stranded DNA. The temperature is then increased to about 70 °C for a special heat-stable polymerase, the TAQ enzyme from *Thermus aquaticus*, to add complementary bases (A, T, G, or C) to the single-stranded DNA and complete the extension to form a new double strand of DNA. This is called a thermal cycle. After this cycle, the tube is heated to 95 °C again for the next cycle. After one thermal cycle, one copy of DNA will become two copies. After about 21 cycles and 31 cycles, 1 million and 1 billion copies of the DNA will be formed, respectively. This entire process can be accomplished in less than an hour in an automatic thermal cycler.

After PCR reactions, one still needs to detect the presence of the PCR products to indicate the presence of the pathogen to be detected. In the original PCR procedure, PCR products were detected by electrophoresis, which is time consuming and laborious. Several new PCR protocols have recently been developed to efficiently report successful PCR reactions.

Mrozinski *et al*. (1998) described an automatic system for screening a family of PCR assays for pathogens, which combines DNA amplification and automated homogeneous detection to determine the presence or absence of specific targets. All primers, polymerase, and deoxyribonucleotide bases necessary for PCR as well as a positive control and an intercalating dye are incorporated into the single tablet. The system works directly from an overnight enrichment of the target organisms. No DNA extraction is required. Assays are available for *Salmonella*, *E. coli* O157:H7, *Listeria*, and *L. monocytogenes*. The system uses an array of 96 blue light emission diodes as the excitation sources and a photomultiplier tube to detect the emitted fluorescent signal indicating successful PCR reactions. This integrated system improves the ease of use of the assay. The inclusivity and exclusivity of this system reach almost 100%, meaning that false-positive and false-negative rates are almost zero. Additionally, this automated system can now be used with assays for the detection of *Cryptosporidium parvum* and *Campylobacter jejuni/coli* and for the

quantitative and qualitative detection of genetically modified organisms in soy and corn (Qualicon, Inc. 2003).

Vishnabhatla *et al*. (2000) described a self-contained PCR system that can report the successful PCR reaction by measuring fluorescence reaction in the experimental chambers. A special molecule is annealed to the single-stranded DNA to report the linear PCR amplification. The molecule has the appropriate sequence for the target DNA. It also has two attached particles. One is a fluorescent particle, and the other is a quencher particle. When the two particles are close to each other, no fluorescence occurs. However, when the TAQ polymerase is adding bases to the linear single strand of DNA, it will break this molecule away from the strand. As this occurs, the two particles will separate from each other, and fluorescence will occur. By measuring fluorescence in the tube, a successful PCR reaction can be determined (Applied Biosystems, Inc. 2003).

A new system using molecular beacon technology has been developed. In this technique, all of the reactions are in the same tube. A molecular beacon is a tailor-made hairpin-shaped hybridization probe. The probe is used to attach to target PCR products. On one end of the probe, a fluorophore is attached; on the other end is a quencher of the fluorophore. In the absence of the target PCR product, the beacon is in a hairpin shape and there is no fluorescence. However, during PCR reactions and the generation of target PCR products, the beacons will attach to the PCR products and cause the hairpin molecule to unfold. As the quencher moves away from the fluorophore, fluorescence will occur, and this can be measured. By using molecular beacons containing different fluorophores, one can detect different PCR products in the same reaction tube and thus perform "multiplex" tests of several target pathogens (Bio-Rad 2003).

One of the major problems of PCR systems is that of contamination of PCR products from one test to another. A system developed by the Pasteur Institute attempts to eliminate PCR product contamination by substituting the base uracil for thymine in the entire PCR protocol. Thus, in the reaction tube, there are adenine, uracil, guanine, and cytosine, but not thymine. During the PCR reaction, the resultant PCR products will be AUGC pairing and not the natural ATGC pairing. After one experiment is completed, a new sample is added to another tube for the next experiment. That tube contains an enzyme called uracil-D-glycosylase (UDG), which will hydrolyse any DNA molecules that contain a uracil base. Therefore, if there is contamination from a previous run, the AUGC-DNA will be destroyed before the beginning of the new run. Before a new PCR reaction, the tube with all reagents is heated to 56 °C for 15 min for UDG to hydrolyse any contaminants. During the DNA denaturation step, the UDG will be inactivated and will not act on the new AUGC-PCR products. This

system can detect *Salmonella*, *Listeria monocytogenes*, and other pathogens (Sanofi Pasteur 2003).

The nucleic acid sequence-based amplification (NASBA) technique has been perfected in recent years for water microbiology. It has advantages over the PCR technologies. Since the target is RNA, it can be used to detect RNA viruses and functional mRNA targets. It is isothermal and thus does not require a thermal cycler for the reaction. It is rapid and sensitive for detection of target molecules.

PCR, NASBA, and related genetic technologies can be powerful tools for water microbiology once the existing problems are solved and the systems are automated. Also, analysts have to be convinced to invest time and money to convert to these technologies for the routine analysis of water microbiology.

23.3.2 Biosensors

There are now a large number of biosensors available for detection of target microorganisms in a variety of food, water, clinical, and industrial samples. Ivnitski *et al*. (1999) provided a comprehensive overview of different physicochemical instrumental techniques for direct and indirect identification of bacteria, including infrared and fluorescence spectroscopy, flow cytometry, chromatography, and chemiluminescence techniques, as a basis for biosensor construction. Biosensor development and application are exciting fields in applied microbiology. The basic idea is simple, but the actual operation is quite complex and involves much instrumentation.

Basically, a biosensor is a molecule or a group of molecules of biological origin attached to a signal recognition material. When an analyte comes in contact with the biosensor, the interaction will initiate a recognition signal that can be reported in an instrument. Many types of biosensors have been developed, including a large variety of enzymes, polyclonal and monoclonal antibodies, nucleic acids, and cellular materials. In some applications, whole cells can also be used as a biosensor. Analytes detected include toxins (e.g., staphylococcal enterotoxins, tetrodotoxins, saxitoxin, and botulinum toxin); specific pathogens (e.g., *Salmonella*, *Staphylococcus*, and *Escherichia coli* O157:H7); carbohydrates (e.g., fructose, lactose, and galactose); insecticides and herbicides; ATP; antibiotics (e.g., penicillins); and others. The recognition signals used include electrochemical (e.g., potentiometry, voltage changes, conductance and impedance, and light addressable); optical (e.g., ultraviolet, bioluminescence, chemiluminescence, fluorescence, laser scattering, reflection and refraction of light, surface plasmon resonance, and polarized light); and miscellaneous transducers (e.g., piezoelectric crystals, thermistor, acoustic waves, and quartz crystal). Eggins (1997), Cunningham (1998), Goldschmidt

(1999), and others have produced excellent review articles and books on biosensors.

Recently, much attention has been directed to the development of "biochips" and "microchips," which may detect a great variety of molecules associated with waterborne and foodborne pathogens. Due to the advancements in miniaturization technology, as many as 50 000 individual spots (e.g., DNA microarrays), each spot containing millions of copies of a specific DNA probe, can be immobilized on a specialized microscope slide. Fluorescent labelled targets can be hybridized to these spots and detected. Biochips can also be designed to detect all kinds of waterborne bacteria by imprinting a variety of antibodies, or DNA molecules, against specific pathogens on the chip for the simultaneous detection of pathogens such as *Salmonella*, *Listeria*, *E. coli*, and *Staphylococcus aureus* on the same chip.

Biochips are an exceedingly important technology in life sciences, and the market value is estimated to reach $5 billion by the middle of this decade. This technology is especially important in the rapidly developing field of proteomics, which requires massive amounts of data to generate valuable information. The development of these biochips and microchips provides an impressive method for obtaining a large amount of information for biological sciences.

23.4 WATER APPLICATIONS: CONCLUSIONS

In regard to waterborne pathogen detection, there are several important issues to consider. These chips are designed to detect minute quantities of target molecules. The target molecules must be free from contaminants before being applied to the chips. In order to utilize these chips, water samples must go through extensive cell amplification (growth in culture) or sample concentration by filtration, separation, absorption, or centrifugation. Particles in a water sample will easily block the channels used in the microfluidic biochips. The necessity of preparing a water sample prior to analysis will not allow the biochips to provide "real-time" detection of pathogens in water at this time. Another concern is viability of the pathogens to be detected by biochips. Monitoring the presence of some target molecule will provide evidence only for the presence or absence of the target pathogen, not for the viability of the pathogen in question. Some form of culture enrichment to ensure growth is still needed in order to obtain meaningful results. The potential of biochips and microarrays for waterborne pathogen detection is great; at this time, however, much more research is needed to make this technology a reality in applied water microbiology.

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