

9

Methods to identify and enumerate frank and opportunistic bacterial pathogens in water and biofilms

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9.1 INTRODUCTION

The vast range of heterotrophic plate count (HPC)-detected bacteria are not considered to be frank or opportunistic pathogens, as discussed elsewhere in this book (chapters 4–7) and in previous reviews (Nichols *et al.* 1995; LeChevallier *et al.* 1999; Velazquez and Feirtag 1999; Szewzyk *et al.* 2000). The purpose of this chapter, however, is to highlight important methodological issues when considering “traditional” and emerging procedures for detecting bacterial pathogens in both water and biofilms, rather than giving specific methods for many pathogens.

The focus of this book is on heterotrophic bacteria; nevertheless, many of the methods discussed can also be directed to other (viral and protozoan) frank and opportunistic pathogens. Furthermore, a number of heterotrophs are thought to cause disease via the expression of virulence factors (Nichols *et al.* 1995), such as the emerging bacterial superantigens (McCormick *et al.* 2001). Hence, pathogen detection is not necessarily one based on particular species, but may take the approach of identifying virulence gene(s), preferably by their active expression (possibly including a range of different genera of bacteria; see virulence factors in Table 9.1). As a consequence, many species contain pathogenic and non-pathogenic strains, so ways to “fingerprint” strains of importance (from the environment and human cases) are also discussed in detail.

Table 9.1. Virulence factors and gene targets to identify waterborne genera¹

Pathogen	Virulence factors	DNA probe ²	PCR ²
<i>Aeromonas hydrophila/A. sobria</i>	Cytotoxic toxin, cytotoxic toxin, enterotoxin, aerolysin asoA, protease, haemolysin, haemagglutinin, acetylcholinesterase	Aer	
<i>Campylobacter jejuni/C. coli/C. lari</i>	Cytolethal distending toxin		
<i>Citrobacter freundii</i>	SLT	SLT2	+
<i>Clostridium difficile</i>	Toxin A		
<i>Clostridium perfringens</i>	Cytotoxic enterotoxin		
Diffusely adherent <i>E. coli</i> DEAC	DA	Daa	
Enteroaggregative <i>E. coli</i> EAggEC	EAST1, AggA	astA, EAggEC	-
Enterohaemorrhagic <i>E. coli</i> VTEC	Vero cytotoxins (O157, H7, intimin & Shiga-like), AE lesions	VT1, VT2, VT2 variants, eae	+
Enteroinvasive <i>E. coli</i> EIEC	Invasion	Ial, paB	+
Enteropathogenic <i>E. coli</i> EPEC	Bundle forming pili, AE lesions 94 kDa OMP	AEF, paB, eae	+
Enterotoxigenic <i>E. coli</i> ETEC	Heat-stable enterotoxin STA, STB, Heat-labile enterotoxin LT	STA1, STA2, STB, LT1, LT2	+
<i>Klebsiella pneumoniae</i>	Heat-stable enterotoxin ST	ST	+
<i>Pseudomonas aeruginosa</i>	Exotoxin A		

Pathogen	Virulence factors	DNA probe ²	PCR ²
<i>Salmonella</i> spp.		spvABC	
<i>Shigella</i> spp.	Shiga toxin gene stx, aerobactin, Group-specific O antigen, superoxide dismutase sodB, invasion genes (virB, ipaABCD, ippI, invGF, invAJKH), intracellular spread gene virG, plasmid antigen gene (ipaH) and expression genes (malA, galU, glpK, kcpA)	stx(1), stx(2), or stx(3)	+
<i>Vibrio cholerae</i>	Cholera toxin	cholera toxins A & B, toxR, toxS, toxT, tcpP, ctx and tcpA	+
<i>Vibrio parahaemolyticus</i>	Haemolysin	thermolabile haemolysin (tlh)	+
<i>Yersinia enterocolitica</i>	Heat-stable enterotoxin yst, lipopolysaccharide O side-chain		+

¹ Adapted from Nichols *et al.* (1995).

² See section 9.6.4 on DNA probes and PCR primers.

To test for specific strains or groups of pathogens, it is important to note that most microbiological procedures consist of the following common method steps: concentration/enrichment, detection and often quantification. Unlike enteric viral or parasitic protozoan pathogens, however, where a concentration of only a few organisms per 100 litres is of potential concern (Rose and Gerba 1991), many of the heterotrophic bacterial pathogens are required in vast numbers to cause disease, with some important exceptions (*Escherichia coli* O157, *Shigella*) (Kothary and Babu 2001). Therefore, an extensive concentration step may be unnecessary for detecting significant heterotrophic bacterial pathogens.

Whether to test the water or solid surface slime (biofilm) has received limited discussion in the literature (Szewzyk *et al.* 2000), although recent work using bacteriophage models has highlighted some important reasons why biofilm testing should be considered (Storey and Ashbolt 2002). For example, biofilms have been shown to sequester phages (and presumably heterotrophic pathogens), theoretically reducing relatively high concentrations in the initial water phase of a distribution system to non-detectable concentrations over short distances (a few kilometres). Hence, sporadic erosion/sloughing of biofilms may result in

health concerns to consumers receiving the distribution water; concerns that would probably be masked if they relied only on water testing.

Lastly, any work on the identification and enumeration of pathogens needs to be put into the context of an overall risk management approach, rather than sole reliance on end-of-pipe testing (Fewtrell and Bartram 2001). Therefore, the methods described below need to be considered within the context of why and where specific pathogens are being tested.

9.2 WATER OR BIOFILM SAMPLING FOR PATHOGENS

Health-related microbial testing has been based on examining water samples for over 100 years (Ashbolt *et al.* 2001). Given that pathogens may accumulate and even grow in biofilms associated with piped or bottled waters (Jones and Bradshaw 1996; Barbeau *et al.* 1998; Buswell *et al.* 1998; Falkinham *et al.* 2001), it is surprising how little effort has been focused on developing routine methods for biofilm sampling. In essence, there is no standard biofilm procedure in the water industry.

Methods have, however, been developed to qualitatively and quantitatively assess biofilm growth *in situ* with experimental coupon devices, including Modified Robbins Devices (MRDs), and various annual reactors (Percival *et al.* 2000). MRDs, developed from an earlier Robbins device (McCoy and Costerton 1982), contain replaceable coupon sampling surfaces, which may make use of a wide range of substrata. MRDs have been used for a range of medical, industrial and environmental applications (Johnston and Jones 1995) and, more recently, water distribution pipes (Kalmbach *et al.* 1997; Ollos *et al.* 1998). Nonetheless, until both the importance of biofilm sampling is recognized and inexpensive methods are developed, pathogen sampling is likely to continue with a heavy bias towards the liquid phase.

In the absence of biofilm pathogen testing protocols, surrogates that indicate biofilm development — and therefore potential for increased health risk — have been instigated for some time and include deteriorating loss in disinfection residual and increasing HPC numbers, ATP levels and/or nitrite concentrations (in chloraminated systems) (Cunliffe 1991). All of these would be expected in biologically unstable water — that is, water that is high in total organic carbon or assimilable organic carbon, warm water, and during periods of stagnant or low flow (LeChevallier *et al.* 1996; van der Kooij 1999).

9.3 CULTURE-BASED (TRADITIONAL) METHODS

The traditional approach for drinking-water microbiology has been the monitoring of water quality using microbial indicator organisms, including so-called “total heterotrophs,” by culture in artificial media (Standing Committee of Analysts 1994; WHO 1996; APHA *et al.* 1998). Such tests are relatively inexpensive and reproducible, yet we know they severely underestimate the total number of heterotrophic bacteria by up to several orders of magnitude (Amann *et al.* 1995; Sartory and Watkins 1999), even with extended incubation times and changes in temperature (Elzanfaly *et al.* 1998).

It has long been recognized that artificial culture media lead to only a very small fraction (0.01–1%) of the total viable bacteria present being detected (Watkins and Xiangrong 1997). Furthermore, introduced bacteria progressively deteriorate in aqueous environments, with some initially able to be grown on selective media (described in Table 9.2), then only on non-selective media (so-called stressed cells), and finally becoming non-culturable (so-called viable but non-culturable [VBNC] if still capable of causing infection) (McFeters 1990; Colwell *et al.* 1996; Cervantes *et al.* 1997). Therefore, despite considerable financial/legal costs associated with culture-based results (and associated quality control methods provided in Table 9.2), application of selective agents in any culture-based method, including those for pathogens, is likely to lead to considerable underestimation of the actual number of potentially infective bacteria present.

One method to overcome the limitation of artificial culture media is the use of living host cells (cell culture) to grow pathogens. Good examples are the co-culture of *Mycobacterium avium* or *Legionella pneumophila*, human pathogens associated with domestic water supplies, with free-living amoebae, such as *Acanthamoeba polyphaga*. Growth may occur by different means, as demonstrated by electron microscopy, with *L. pneumophila* residing within the cysts and *M. avium* within the outer walls of the double-walled cysts of *A. polyphaga* (Steinert *et al.* 1998). Furthermore, these locations may provide a reservoir for the bacteria when environmental conditions become unfavourable and allow for inactive pathogens to accumulate with amoebae/cysts in biofilms (Brown and Barker 1999). In addition to various amoebae, the nematode *Caenorhabditis elegans* may prove to be a suitable host for detecting a range of pathogens (Labrousse *et al.* 2000).

9.4 CONCENTRATION OF TARGET BACTERIA

Heterotrophic bacteria are traditionally concentrated/trapped on membrane filters with porosities of 0.22–0.45 μm or enriched by selective growth. In some

Table 9.2. International standardization of methods for microbiological drinking-water analyses¹

Target organisms	ISO standard	Culturing technique, medium/media and incubation	Observations
<i>Legionella</i> species	ISO 11731	Spread plating on GVPC medium with antibiotics at 36 °C for 10 days; subculturing on BYCE and BCYE-cys; serological testing of isolates growing on BYCE but not on BCYE-cys; identification by fatty acids, isoprenoid quinones, indirect or direct immunofluorescent antibody assay, slide or latex bead agglutination, genus-specific monoclonal antibody or enzyme-linked immunosorbent assay	With and without sample pretreatment; background growth interferes; antibiotics and identification increase costs
<i>Legionella</i> species	(ISO 11731-2)	A screening method based on membrane filtration	
<i>Pseudomonas aeruginosa</i>	[ISO 8360-2]	Membrane filtration on Drake's medium 19, incubation at 37 °C for 2 days; for confirmation subculturing on milk agar at 42 °C for 1 day (growth, casein hydrolysis, fluorescence and pyocyanine)	Atypical isolates should be further identified; material not expensive but labour costs significant
<i>Pseudomonas aeruginosa</i>	[ISO 8360-1]	Liquid culturing in Drake's medium 10 at 37 °C for 2 days; for confirmation subculturing on milk agar at 42 °C for 1 day (growth, casein hydrolysis, fluorescence and pyocyanine)	Atypical isolates should be further identified; material not expensive, but labour costs significant
<i>Salmonella</i> species	[ISO 6340]	Liquid pre-enrichment in buffered peptone water at 36 °C for 1 day, enrichment in modified Rappaport-Vassiliadis broth at 42 °C for 1 day, selection on brilliant green/phenol red lactose and xylose lysine deoxycholate agar at 36 °C for 1 day and optionally on bismuth sulfite agar at 36 °C for 2 days; isolation of typical colonies for confirmation using biochemical and serological tests	<i>S. typhi</i> needs another pre-enrichment medium; time and many media needed, which increases costs

Target organisms	ISO standard	Culturing technique, medium/media and incubation	Observations
Staphylococci	CEN/TC 230	Membrane filtration	Recently started activity
Total heterotrophs	ISO 6222	Pour plate technique, yeast extract agar, incubation at 36 °C for 2 days and at 22 °C for 3 days	All microorganisms are not expected to generate colonies; changes in cfu relevant; cheap method
Evaluation of membrane filters	ISO 7704	Comparison of relative recoveries for a method	
Evaluation of colony count media	ISO 9998	Comparison of relative recoveries for a method	
Validation of microbiological cultivation methods	(ISO TR 13843)	Characterization of methods and confirmation of the detection of the target organism	
Equivalence testing of microbiological cultivation methods	(ISO 17994)	Comparison of relative recoveries of target organisms between different methods	

¹ ISO numbers refer to a published standard, () standard proposal not yet published or [] published standard under revision, taken from Köster *et al.* (2002).

instances, as for the motile *Campylobacter*-like organisms, motile species are first selected for by active movement through a larger-porosity filter (e.g., 0.6 µm) directly over the enrichment medium (Steele and McDermott 1984). Membrane filtration is also recommended as the concentration step prior to direct molecular identification (see below). It should be recognized that there are many bacterial species known to be able to pass through 0.45-µm membranes, some of which may well be opportunistic pathogens, such as various mycobacteria (Marolda *et al.* 1999) (hence the recommendation to use 0.2-µm membranes). Furthermore, by definition, bacteria that are <0.3 µm in diameter and do not significantly increase in size when inoculated onto a nutrient-rich medium are called ultramicrobacteria (Torrella and Morita 1981). The relevance of bacterial pathogens that pass through a 0.2-µm membrane (other than the cell wall-less groups) has largely been ignored.

9.5 GROWTH AND DETECTION WITH CHROMOGENIC SUBSTANCES

In addition to ISO methods for detection of pathogens from waters, which generally rely on selective enrichments followed by secondary culture and biochemical testing for confirmations (Table 9.2), research for more reliable and faster methods continues. One result is the use of chromogenic compounds, which may be added to the conventional or newly devised media used for the isolation of heterotrophs. These chromogenic substances are modified either by enzymes that are typical for the respective bacteria or by specific bacterial metabolites. After modification, the chromogenic substance changes its colour or its fluorescence, thus enabling easy detection of those colonies displaying the metabolic capacity. In this way, these substances can be used to avoid the need for isolation of pure cultures and confirmatory tests. The time required for the determination of different bacteria can be cut down to 18–14 h, which makes results available the next working day.

Currently, a number of different media based on enzyme-specific tests have been developed for pathogens (Carricajo *et al.* 1999; Perry *et al.* 1999; Karpiskova *et al.* 2000) and are becoming routine in clinical and food laboratories. These media allow detection, enumeration and identification to be performed directly on the isolation plate or in the broth. In general, four groups of fluorogenic and chromogenic compounds can be distinguished: fluorogenic dyes, pH-fluorescent indicators, redox indicators and enzyme substrates. Such tests could be equally well applied to water or biofilm homogenates, although there are few comparisons specifically discussed in the current literature, except for faecal indicators (Manafi 1999).

9.6 IMMUNOLOGICAL AND NUCLEIC ACID-BASED METHODS

A range of biochemical-based detection methods have developed over the last 20 years. These were initially based solely on antibodies and more recently in combination with nucleic acid-based approaches. Each of these is now discussed.

9.6.1 Antibody-based methods

Antibodies, glycoproteins produced by mammals as part of their defence system against foreign matter, possess highly specific binding and recognition domains

that can be targeted to specific surface structures of a pathogen (antigen). Antibody techniques used to detect a wide range of pathogens in clinical, agricultural and environmental samples are referred to as immunological methods.

Antisera or polyclonal antibodies are the original source of immune reagents; they are obtained from the serum of immunized animals (typically rabbits or sheep). The preparations comprise a mixture of antibody molecules each with different reactivities (affinities and specificities) for the immunized material, and the response to immunization varies between animals and between bleeds from the same animal. Monoclonal antibodies, produced *in vitro* by fusing plasma cells of an immunized animal (usually a mouse or rat) with a cell line that grows continuously in culture, so that the fused cells will grow continuously and secrete only one kind of antibody molecule (Goding 1986), can be much better standardized and generally give greater specificity than polyclonal antibodies (Torrance 1999).

For example, monoclonal antibodies have been successfully used for the detection of campylobacters (Buswell *et al.* 1998), *E. coli* O157:H7 (Tanaka *et al.* 2000), *Helicobacter pylori* (Hegarty *et al.* 1999), *Legionella* (Steinmetz *et al.* 1992; Obst *et al.* 1994) and mycobacteria (Wayne *et al.* 1996). Viable cells may be detected with antibodies if precultivated in a selective medium to raise the number up to detectable numbers, so avoiding (the possible complication of) detecting dead cells. Another option for the detection of “viable” heterotrophs is the combination of immunofluorescence (IF) with a respiratory activity compound (such as cyanoditolyl tetrazolium chloride, or CTC). An IF/CTC approach has been described for the detection of *E. coli* O157:H7, *Salmonella typhimurium* and *Klebsiella pneumoniae* in water (Pyle *et al.* 1995). In general, immunological methods can easily be automated in order to handle high sample numbers and often form the basis of pathogen biosensors (outlined in section 9.5).

A more traditional use of antibodies is their conjugation to latex beads and interaction with the target antigen, in what are called antibody agglutination assays, to confirm the presence of particular pathogens following culture. In the confirmation of *E. coli* O157:H7, for example, negative sorbitol-fermenting colonies after growth on sorbitol MacConkey agar are screened by antibody agglutination (Taormina *et al.* 1998).

9.6.2 Immunomagnetic separation

Immunomagnetic separation (IMS) offers an alternative approach to rapid identification of culturable and non-culturable microorganisms (Safarik *et al.* 1995). The principles and application of the method are simple but reliant on

suitable antibody specificity under the conditions of use. Purified antigens are typically biotinylated and bound to streptoavidin-coated paramagnetic particles. The raw sample is gently mixed with the immunomagnetic beads; then, a specific magnet is used to hold the target organisms against the wall of the recovery vial, and non-bound material is poured off. If required, the process can be repeated, and the beads may be removed by simple vortexing. Target organisms can then be cultured or identified by direct means.

The IMS approach has been applied to the recovery of *E. coli* O157 from water (Anonymous 1996), and commercial kits utilizing IMS concentration of pathogens are available. Furthermore, *E. coli* O157 detection following IMS can be improved by electrochemiluminescence detection (Yu and Bruno 1996) or solid-phase laser cytometry (Pyle *et al.* 1999). It is important to note, however, that false-negative detection by IMS may occur due to the loss of surface antigen properties from the target cells via environmental decay and induced by starvation, as shown for *E. coli* O157:H7 (Hara-Kudo *et al.* 2000). Nonetheless, IMS may also detect VBNC cells (Velazquez and Feirtag 1999). IMS is probably best used in combination with gene amplification and probing methods, which are discussed next.

9.6.3 Gene sequence-based methods

Advances in molecular biology in the past 20 years have resulted in a number of new detection methods that depend on the recognition of specific gene sequences. Such methods are usually rapid and can be tailored to detect specific strains of organisms on the one hand or groups of organisms on the other. The methods have a substantial potential for future application in the field of drinking-water hygiene (Havelaar 1993). An international expert meeting in Interlaken concluded (OECD 1999) that the application of molecular methods was currently largely limited to research, verification and outbreak investigation, and that its usefulness in routine monitoring remained to be proven. These new methods are largely based around the polymerase chain reaction (PCR) and gene sequence pattern (“fingerprint”) identification approaches described below. To date, they have largely impacted on epidemiology and outbreak investigations rather than the routine testing of finished drinking-water.

9.6.4 Polymerase chain reaction

With the PCR and two suitable primer sequences (fragments of nucleic acid that specifically bind to the target organism), trace amounts of DNA can be

selectively multiplied. In principle, a single copy of the respective sequence in the assay can produce over a million-fold identical copies, which can then be detected and further analysed by different methods. Examples of genes used for the specific detection of various pathogens are listed in Table 9.1; however, for the identification of different taxa, the 16S and 23S ribosomal RNA (rRNA) genes are often the most useful (Olsen *et al.* 1986; Szewzyk *et al.* 1994). Furthermore, a range of methods have been developed for the purification of nucleic acids from the environment, including bispeptide nucleic acids (bis-PNAs; PNA clamps), PNA oligomers and DNA oligonucleotides as affinity purification reagents for sub-femtomoles per litre 16S ribosomal DNA (rDNA) and rRNA targets. The most efficacious capture system depends upon the particular sample type (and background nucleic acid concentration), target (DNA or RNA) and detection objective (Chandler *et al.* 2000).

One problem faced with the PCR test is the low volume assayed, in the order of some microlitres, whereas the water sample volume is in the range of 100 ml to 100 litres — hence the need to prefilter and/or IMS concentrate the target organism(s). A resulting problem, however, is that natural water samples often contain substances (like humic acids and iron) that may also concentrate and subsequently interfere with the PCR. Hence, it is critical to have positive and negative controls with each environmental sample PCR to check for inhibition and specificity.

In addition to control samples in PCR runs, subsequent sequence analysis or hybridization of the product amplicon with a second specific probe can greatly reduce the probability of false-positive detection of (non-target) organisms. In the detection of the genus *Mycobacterium* by PCR targeting the 16S rDNA, the specificity and sensitivity of such a two-step method were confirmed with various target and non-target reference strains, followed by application in native biofilms from different drinking-water distribution systems (Schwartz *et al.* 1998). The results of the Schwartz *et al.* (1998) investigation showed that mycobacteria could not be detected when groundwater was used as raw water source, but were frequently found in bank-filtered drinking-water biofilms. Importantly, further PCR experiments indicated that the detected mycobacteria did not belong to the pathogenic or certain opportunistic pathogenic species of this genus, but were representatives of the environmental mycobacteria.

Various hybridization probes are available, are easy to implement and are far more rapid than conventional biochemical confirmation methods. For example, a rapid hybridization protocol for *Campylobacter jejuni*, utilizing a 1475-bp chromogen-labelled DNA probe (pDT1720), was developed by L.-K. Ng *et al.* (1997) for food samples. Based on the nucleotide sequence of pDT1720, a pair of oligonucleotide primers was also designed for PCR amplification of DNA from *Campylobacter* spp. after overnight growth in selective Mueller-Hinton

broth with cefoperazone and growth supplements. All *C. jejuni* strains tested, including deoxyribonuclease-producing strains and *C. jejuni* subsp. *doylei*, produced the specific 402-bp amplicon, as confirmed by restriction and Southern blot analysis. The detection range of the assay was as low as 3 cfu per PCR to as high as 10^5 cfu per PCR for pure cultures.

The generally greater sensitivity of PCR over conventional culture-based methods is often suggested to be due to the detection of naked nucleic acids, living microorganisms and dead microorganisms (Toze 1999). One way to resolve these various targets is to use a short (e.g., 3 h) preincubation period in a selective medium so that only growing organisms are detected (Frahm *et al.* 1998). Other options include the use of nested PCR (second primer set targeting regions within the first set's amplicon) (Guimaraes-Peres *et al.* 1999) or multiplex PCR (targeting two different genomic regions in the one reaction) (Campbell *et al.* 2001).

Also under development are methods targeting short-lived nucleic acids, such as messenger RNA or rRNA (Sheridan *et al.* 1998). Nonetheless, false negatives can occur, as illustrated in the analysis of legionellae from 80 cooling tower water samples using both cultural and PCR methods (D.L.K. Ng *et al.* 1997). D.L.K. Ng *et al.* (1997) performed the PCR with the Perkin Elmer EnviroAmp *Legionella* kit, and 47 samples (58.8%) appeared positive by both methods; 29 samples (36.3%) were positive by PCR only, while 4 samples (5%) showed PCR inhibition despite the adoption of the more stringent sample preparation protocol especially designed to eliminate inhibitors.

A most important advantage of PCR is that the target organism(s) do not need to be culturable. Detection of novel unculturable pathogens has resulted from the use of PCR, such as the finding of *Gastrospirillum hominis* by cloning its 16S rRNA into *E. coli* and subsequent sequence analysis (Solnick *et al.* 1993). Based on its 16S rDNA sequence, this unculturable *Helicobacter*-like organism appeared closely related to *H. felis* and may be the only *Helicobacter*-like bacterium to infect humans and small animals.

PCR is of particular advantage for the analysis of pathogens among high numbers of background bacteria in pipe biofilms, such as *Mycobacterium* spp. and *Helicobacter pylori*, which are difficult to culture and, in the case of *Mycobacterium*, not different at the 16S rRNA level (Roth *et al.* 1998; Mackay *et al.* 1999). For example, Mackay *et al.* (1999) used an MRD incorporating removable stainless steel coupons to investigate the persistence of *H. pylori* in mixed-species heterotrophic laboratory biofilms. While dead (heat-inactivated) *H. pylori* (NCTC 11637) did not persist in the biofilm, live cells were detected in biofilm material well after theoretical washout. Hence, Mackay *et al.* (1999)

suggested that the organism possessed the ability to persist in the mixed-species heterotrophic biofilm and may pose a risk to public health.

Rapid improvements in *in situ* labelling methods have facilitated the development of a number of quantitative PCR approaches, which can be achieved by real-time PCR machines — those that follow amplicon production during the PCR cycles. Pathogens can be identified in as little as 30 min in commercially available real-time PCR machines (Cockerill and Smith 2002). *In situ* detection of amplicons is generally by fluorescent reporter probes, such as those in molecular beacons (which fluoresce only when the quencher and fluorochrome are separated) or by fluorescent resonance energy transfer (pumping of one fluorochrome by the emission of another).

9.6.5 Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) uses gene probes with a fluorescent marker, typically targeting the 16S rRNA (Amann *et al.* 1995). Concentrated and fixed cells are permeabilized and mixed with the probe. Incubation temperature and addition of chemicals can influence the stringency of the match between the gene probe and the target sequence. Since the signal of a single fluorescent molecule within a cell does not allow detection, target sequences with multiple copies in a cell have to be selected (e.g., there are 10^2 – 10^4 copies of 16S rRNA in active cells). A number of FISH methods for the detection of coliforms and enterococci have been developed (Meier *et al.* 1997; Fuchs *et al.* 1998; Patel *et al.* 1998), but few fluorescent oligoprobes have been developed for waterborne bacterial pathogens or their environmental hosts (Grimm *et al.* 2001).

Although controversial for many pathogens, low-nutrient environments may result in cells entering a non-replicative VBNC state (Bogosian *et al.* 1998). Such a state not only may give us a false sense of security when reliance is placed on culture-based methods, but also may give the organisms further protection (Lisle *et al.* 1998; Caro *et al.* 1999). An indication of VBNC *Legionella pneumophila* cell formation was given by following decreasing numbers of bacteria monitored by colony-forming units, acridine orange direct count and hybridization with 16S rRNA-targeted oligonucleotide probes (Steinert *et al.* 1997). It was therefore concluded that FISH detection-based methods may better report the presence of infective pathogens and viable indicator bacteria. Yet cells may remain FISH-positive for two weeks after cell death, so inclusion of some activity stain (e.g., CTC) is necessary to confirm viability (Prescott and Fricker 1999).

A further extension of the FISH approach to improve signal strength is the use of peptide nucleic acid probes targeted against the 16S rRNA molecule,

such as used to detect *E. coli* from water (Prescott and Fricker 1999). The probe was labelled with biotin, which was subsequently detected with streptavidin horseradish peroxidase and the tyramide signal amplification system. *E. coli* cells were concentrated by membrane filtration prior to hybridization and the labelled cells detected by a commercial laser-scanning device within 3 h. Detection and enumeration of labelled pathogens are also possible by the use of a flow cytometer (Fuchs *et al.* 1998; Tanaka *et al.* 2000). Nonetheless, the main limitation with flow cytometry is the often low signal-to-noise ratio between FISH-labelled cells and background autofluorescence of environmental samples (Deere *et al.* 2002).

9.7 FINGERPRINTING METHODS

Over the last 20 years, a diversity of fingerprinting methods has arisen. Analysis of isoenzymes, serotyping and, more recently, macrorestriction analysis (using pulsed field gel electrophoresis [PFGE]) are well established methods for the typing of bacterial pathogens (Jonas *et al.* 2000). Due to the necessary skills, expensive equipment or access to a collection of monoclonal antibodies, however, these are often restrictive approaches. Multilocus enzyme electrophoresis (MLEE) is the preferred enzyme analysis method, which estimates the overall genetic relatedness among strains by indicating allele variation in a random sample of chromosomally encoded metabolic housekeeping enzymes (Selander *et al.* 1986). In general, however, one would expect MLEE analysis to be less discriminating between pathogenic and non-pathogenic strains than direct PCR amplification and sequencing of putative toxin genes (Nachamkin *et al.* 2001). Hence, application of DNA-based techniques for culturable and non-culturable cells based on ribotyping, RNA profiling and various PCR-based DNA fingerprinting methods, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP) and PFGE, is now outlined.

9.7.1 Ribotyping

Ribotyping is a well regarded method of genotyping pure culture isolates and is often used in epidemiological studies. The basis of ribotyping is the use of rRNA as a probe to detect chromosomal RFLPs. Hence, the whole DNA of a pure culture is extracted, cleaved into various length fragments by the use of one of many endonucleases, which are separated by gel chromatography, then

probed with labelled rRNA oligonucleotides (oligos) (Southern blot analysis). Although ribotyping is accurate, it is time and labour consuming, not only needing pure isolates, but also requiring the undertaking of Southern blot analysis, all of which are now possible with automated machines.

A modification of the method is PCR-ribotyping, which uses PCR to directly amplify the 16S–23S intergenic spacer region of the bacterial rRNA operon. The heterogeneity in the length of the spacer region allows for an alternative to standard ribotyping, as illustrated for *Burkholderia (Pseudomonas) cepacia* (Dasen *et al.* 1994). PCR-ribotyping has been shown to be a rapid and accurate method for typing a range of bacteria and is less labour intensive than standard ribotyping (Dasen *et al.* 1994).

9.7.2 Profiling of low-molecular-weight RNA

Direct detection of the diversity of bacteria from the environment is also possible. A method developed over 20 years ago is the profiling of low-molecular-weight (LMW) RNA (5S rRNA and transfer RNA [tRNA]) (Höfle 1998). The technique is straightforward; total RNA (23S, 16S and 5S rRNA, as well as tRNA present in high copy number in viable cells) is extracted from an environmental sample and separated by high-resolution polyacrylamide gel electrophoresis. The separation profiles of the 5S rRNA and tRNA (the 23S and 16S rRNA are too big to enter the gel) can be visualized by silver staining or by autoradiography if the RNA is radioactively labelled. Subsequently, the profiles are scanned and stored in an electronic database for comparison.

For example, LMW RNA profiling was used to monitor bacterial population dynamics in a set of freshwater mesocosms after addition of non-indigenous bacteria and culture medium (Höfle 1992). The addition of the bacteria had no effect on the indigenous bacterioplankton. However, the added culture medium caused an increase of two of the natural bacterial populations — namely, a member related to *Aeromonas hydrophila* and bacteria related to *Cytophaga johnsonae*. Hence, LMW RNA profiling may allow the direct detection of specific genera and sometimes bacterial species within aquatic environments.

Further resolution with LMW rRNA fingerprinting can be achieved by using DGGE (described below). There are practical limitations, however; rRNA rapidly degrades, forming additional bands in the profiles (Stoner *et al.* 1996). Furthermore, the small size of the different LMW RNAs (5S rRNA maximal 131 nucleotides, and tRNA maximal 96 nucleotides) limits their phylogenetic information (limits discrimination to general or above).

9.7.3 Restriction fragment length polymorphism

In traditional RFLP analysis, DNA is isolated from pure culture isolates and subject to specific cleavage by one or more endonucleases before separating the fragments by gel chromatography. To improve the resolution between DNA fragments, restriction enzyme analysis can be followed by PFGE (Schoonmaker *et al.* 1992).

Schoonmaker *et al.* (1992) compared ribotyping and restriction enzyme analysis by PFGE for *L. pneumophila* isolates from patients, their environment and unrelated control strains during a nosocomial outbreak. Two of the patterns were observed in the three *L. pneumophila* serogroup 6 isolates from patients with confirmed nosocomial infections and environmental isolates from the potable water supply, which was, therefore, believed to be the source of the patients' infections. Additional pattern types from patients with legionellosis were seen in isolates from the hospital environment, demonstrating the presence of multiple strains in the hospital environment. While both techniques successfully subtyped the isolates obtained during the investigation of the outbreak, restriction enzyme analysis by PFGE was useful for subdividing ribotypes and for distinguishing strains involved in the outbreak from epidemiologically unrelated strains (Schoonmaker *et al.* 1992).

A rapid two-step identification scheme based on PCR-RFLP analysis of the 16S rRNA gene was developed in order to differentiate isolates belonging to the *Campylobacter*, *Arcobacter* and *Helicobacter* genera. For 158 isolates (26 reference cultures and 132 clinical isolates), specific RFLP patterns were obtained, and species were successfully identified by this assay (Marshall *et al.* 1999). Furthermore, a novel helicobacter, *Helicobacter canadensis*, was distinguished from *H. pullorum* by RFLP analysis using the restriction enzyme ApaLI (Fox *et al.* 2000).

It should be noted, however, that the success of RFLP analysis is organism or group specific, and trialling of many restriction enzymes can be frustrating. For example, Smith and Callihan (1992) were able to correctly identify *Bacteroides fragilis* strains, but were unable to generate RFLPs that could be used to specifically separate enterotoxin-producing strains from non-enterotoxigenic strains (Smith and Callihan 1992).

There are also practical issues associated with comparing between gels, particularly for PFGE, in that small changes in running conditions may yield different results. Hence, software designed to reduce misclassifications has been developed (Wang *et al.* 2001).

9.7.4 Amplified fragment length polymorphisms and arbitrarily primed PCR

DNA RFLPs are extremely valuable tools for laboratory-based evaluation of hypotheses generated by epidemiological investigations of infectious disease outbreaks. Using PCR-typing protocols, however, provides the advantage that minute amounts of target DNA can be analysed in a very short time. Numerous PCR-based typing protocols have been introduced, which take advantage of rapid screening of these small volumes, such as random amplification of genomic DNA (Welsh and McClelland 1990). Two types of PCR-based subtyping methods are preferred (Jonas *et al.* 2000). The PCR-RFLP method involves the amplification of previously characterized or phylogenetically conserved targets followed by restriction endonuclease analysis to evaluate polymorphisms within the amplified sequences (called AFLP or amplified 16S rDNA restriction analysis [ARDRA]). The second approach uses random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR (AP-PCR), which require no prior knowledge of DNA sequences of test organisms because they rely on random amplification of target DNA by arbitrarily chosen primers. In addition, RAPD and AP-PCR do not require any restriction analysis of amplified DNA, as fragments are the product of the PCR.

There are several issues to be recognized with PCR-based methods. The approaches are organism dependent and do not always provide adequate discrimination between unrelated isolates. RAPD methods often may suffer from poor reproducibility, particularly when the amplification is performed by using crude target DNA under non-stringent conditions (Tyler *et al.* 1997). Hence, it is important to have protocols that are defined and followed strictly (Grundmann *et al.* 1997). Lastly, fingerprinting patterns from AP-PCR or RAPDs are often complex and at best have to be analysed by means of DNA sequencing gels, using automated laser fluorescence analysis systems, and compared unambiguously with specialized software (Grundmann *et al.* 1995).

In an analysis of 29 outbreak-associated and 8 non-associated strains of *Legionella pneumophila*, Jonas *et al.* (2000) demonstrated that *Sfi*I macrorestriction analysis, AFLP and AP-PCR all detected one predominant genotype associated with the outbreaks in hospitals. All of them correctly assigned epidemiologically associated environmental isolates to their respective patient specimens. Although AP-PCR was the least discriminating and least reproducible technique, it demonstrated the best interassay reproducibility (90%) and concordance (94%) in comparison with the genotyping standard of macrorestriction analysis and the epidemiological data. Analysis of AFLP fragments revealed 12 different types and subtypes. Hence, because of its

simplicity and reproducibility, AFLP proved to be the most effective technique in outbreak investigation (Jonas *et al.* 2000).

9.7.5 Repetitive gene PCR

PCR-mediated amplification of regions bordered by enterobacterial repetitive intergenic consensus sequences or repetitive extragenic palindrome motifs have proved to be valuable tools to examine genetic variation among an extensive range of bacterial species (Versalovic *et al.* 1991). However, relatively relaxed primer annealing conditions have been used in these studies, and it remains to be determined whether the enterobacterial repetitive intergenic consensus and repetitive extragenic palindrome PCRs are basically different from AP-PCR or analysis by RAPD (Welsh and McClelland 1990).

Consequently, a high-stringency PCR assay, targeting regions within the various bacterial genomes and bordered by invertedly repeated elements (known as a “BOX”) (Martin *et al.* 1992), have been developed (BOX-PCR) (Kainz *et al.* 2000). For example, 15 strains of *Salmonella enterica* subsp. *enterica* serotypes Typhi (10), Paratyphi A (1) and Typhimurium (3) collected over a period of 15 years from stool, blood and urine samples and the Ganga River were tested by ARDRA, RAPD and BOX-PCR methods (Tikoo *et al.* 2001). In ARDRA, strains belonging to the same species were identified by identical fingerprints; RAPD, on the other hand, divided *Salmonella* into nine different groups. In BOX-PCR, all the strains of *Salmonella* showed six different groups, but with the presence of a common band. It was observed that RAPD had higher discriminatory power than BOX-PCR and was a simple and rapid technique for use in epidemiological studies of isolates belonging to *S. enterica* (Tikoo *et al.* 2001).

Nonetheless, for reasons discussed below, RAPD is generally not preferred, and genetic typing methods using repeating intergenic DNA and PCR (Gillings and Holley 1997; Dombek *et al.* 2000) and ribotyping (Parveen *et al.* 1999; Carson *et al.* 2001) should be considered first.

9.7.6 Denaturing and temperature gradient gel electrophoresis

In applications where pure cultures are either not available or not wanted, Muyzer and co-workers introduced a genetic fingerprinting technique directed to microbial ecology, which is based on DGGE (Myers *et al.* 1987) of PCR-amplified 16S rRNA fragments (Muyzer *et al.* 1993). The method is rapid and straightforward and does not depend on expensive equipment. Mixtures of PCR

products obtained after enzymatic amplification of genomic DNA extracted from a complex assemblage of microbes are separated in polyacrylamide gels containing a linear gradient of DNA denaturants (urea and formamide) (Muyzer and Smalla 1998). Sequence variation among the different DNA molecules influences the melting behaviour, and therefore molecules with different sequences will stop migrating at different positions in the gel.

Another technique based on the same principle is TGGE (Riesner *et al.* 1991), which can also be applied to separate 16S rDNA fragments. While non-culturable environmental bacteria can be detected, the approach relies upon linking rDNA from community fingerprints to pure culture isolates from the same habitat. For example, digoxigenin-labelled polynucleotide probes can be generated by PCR, using bands excised from TGGE community fingerprints as a template, and applied in hybridizations with dot blotted 16S rDNA amplified from bacterial isolates (Muyzer and Smalla 1998). Within 16S rDNA, the hypervariable V6 region, corresponding to positions 984–1047 (*E. coli* 16S rDNA sequence), which is a subset of the region used for TGGE (positions 968–1401), best met the criteria of high phylogenetic variability, required for sufficient probe specificity, and closely flanking conserved priming sites for amplification. Removal of banking conserved bases was necessary to enable the differentiation of closely related species. This was achieved by 5' exonuclease digestion, terminated by phosphorothioate bonds that were synthesized into the primers. The remaining complementary strand was removed by single-strand-specific digestion. Standard hybridization with truncated probes allowed differentiation of bacteria that differed by only two bases within the probe target site and 1.2% within the complete 16S rDNA.

9.7.7 Single-strand conformation polymorphism

An alternative to sequencing is SSCP analysis, developed in 1989 (Orita *et al.* 1989), which uses small sequences of a target gene that has been amplified by PCR. Fragments are heat denatured to create single strands, and the single strands are subsequently renatured, causing the strands to adopt “tertiary” conformations based on their base sequences. Thus, fragments with different base sequences have different conformations. For analysis, these fragments are separated by electrophoresis with a non-denaturing gel, in which each fragment will consistently travel at a unique rate even when fragments are of identical size.

SSCP was primarily designed to detect sequence mutations, including single base substitutions, in genomic DNA (Orita *et al.* 1989). SSCP analysis of PCR-amplified fragments of the 16S rRNA gene has more recently been used as an alternative to genomic sequencing for the identification of bacterial species

(Ghozzi *et al.* 1999). The use of a fluorescence-based capillary electrophoresis system for SSCP analysis has also contributed to the efficiency of the methodology and reliability of analysis (Gillman *et al.* 2001). Gillman *et al.* (2001) used multiple-fluorescence-based PCR and subsequent SSCP analysis of four variable regions of the 16S rRNA gene to identify species-specific patterns for 30 of the most common mycobacterial human pathogens and environmental isolates.

9.8 EMERGING METHODS

Current developments in high-throughput nucleic acid sequencing and so-called “gene chip” or microarray technology are having a major impact on bacterial epidemiology. Whole genome sequencing is now a reality, facilitating the identification of novel target sequence motifs for epidemiological typing. Multilocus sequence typing (MLST) is one example, being the sequence polymorphism detected in a number of slowly evolving genes and providing for the categorization of strains on the basis of allelic diversity (Dingle *et al.* 2000; Blackwell 2001; McGee *et al.* 2001). MLST is a development of multilocus enzyme electrophoresis in which the alleles at multiple housekeeping loci are assigned directly by nucleotide sequencing, rather than indirectly from the electrophoretic mobilities of their gene products. A major advantage of this approach is that sequence data are unambiguous and electronically portable, allowing molecular typing of bacterial pathogens (or other infectious agents) via the Internet. Hence, MLST should also be a good method for discriminating between different virulence factors.

DNA chip or microarray technology is characterized by high-throughput probe-mediated nucleic acid identification capacity. In contrast, biosensors in the medical area have largely been based on antibody technology, the antigen triggering a transducer or linking to an enzyme amplification system. Biosensors based on gene recognition, however, are looking very promising in the microarray format for detecting and even quantifying microorganisms (Cho and Tiedje 2002).

As little material can actually come in contact with the microarray, ways to concentrate water samples will be necessary, such as membrane filtration or IMS concentration. Nonetheless, detection limits are currently not very sensitive: direct plating of washed IMS beads showed a positive recovery of *E. coli* O157:H7 directly from poultry carcass rinse at an inoculum of 10 cfu/ml, whereas IMS used with direct PCR amplification and microarray detection gave a process-level detection limit (automated cell concentration through microarray detection) of $<10^3$ cfu/ml in poultry carcass rinse water (Chandler *et al.* 2001).

There are two variants of the DNA microarray technology, in terms of the property of arrayed DNA sequence with known identity:

- Probe cDNA (500–5000 bases long) is immobilized to a solid surface such as glass using robot spotting and exposed to a set of targets either separately or in a mixture. This method, “traditionally” called DNA microarray, is widely considered to have been developed at Stanford University (Ekins and Chu 1999).
- An array of oligonucleotides (20–25 bases long) or PNA probes is synthesized either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to labelled sample DNA and hybridized, and the identity and abundance of complementary sequences are determined (Lemieux *et al.* 1998; Lipshutz *et al.* 1999) (Figure 9.1).

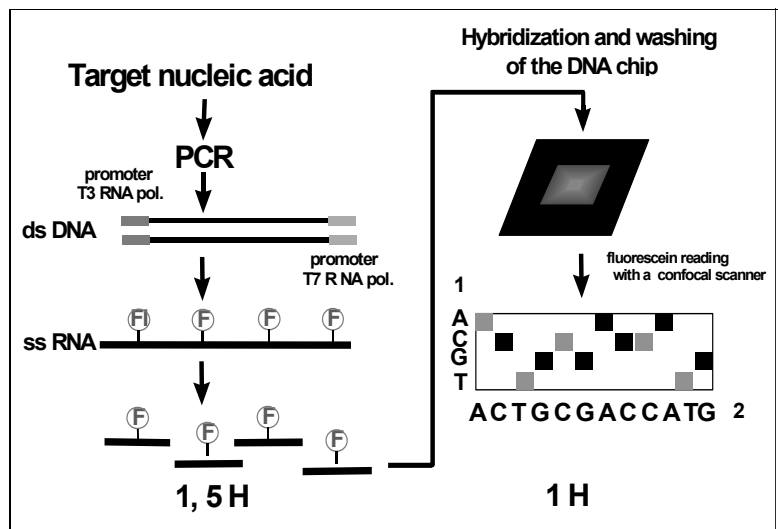


Figure 9.1. Test format of the DNA chip array. Nucleic acid extraction, amplification and labelling are manual steps, whereas hybridization, DNA chip reading and interpretation are undertaken by dedicated instrumentation (ds DNA: double-stranded DNA; ss RNA: single-stranded RNA; F: labelled ribonucleotide (rUTP-fluoresceine); 1: nucleotide tested at interrogation position; 2: nucleotide at interrogation position). Source: www.affymetrix.com.

The vast amount of information captured with microarrays necessitates skills in bioinformatics as well as in microarray technology and microbiology (Wu *et al.* 2001), although various commercial products (see, for example, <http://www.esd.ornl.gov/facilities/genomics/equipment.html>) are now available for preparing and reading the results from microarrays. Perhaps seen as fortuitous, the current interest in molecular recognition of biowarfare agents has greatly hastened developments in new and improved biosensors using a range of molecular recognition components (e.g., antibody, aptamer, enzyme, nucleic acid, receptor, etc.) (Iqbal *et al.* 2001). Improvements in the affinity, specificity and mass production of the molecular recognition components may ultimately dictate the success or failure of detection technologies in both a technical and commercial sense, as discussed in the excellent review by Iqbal *et al.* (2001). Achieving the ultimate goal of giving the individual soldier on the battlefield or civilian responders to an urban biological attack or epidemic a miniature, sensitive and accurate biosensor may depend as much on molecular biology and molecular engineering as on hardware engineering.

9.9 CONCLUSIONS

A major limitation to our understanding the full extent of bacterial pathogens in aquatic environments has been our limited understanding of the majority of bacterial types present, when culture-based methods have been applied. Current methods are still heavily reliant on growing cells in media, but these have been significantly improved by the application of chromogenic substances that detect specific bacterial metabolites. We are now, however, able to dislodge cells from biofilms and fractionate bacteria or filter them from waters prior to direct detection or an amplification process. The most important molecular amplification process is that of the PCR. Not only can we detect non-culturable bacteria, but, coupled with various PCR product-separating techniques, highly specific fingerprinting of different strains of bacteria is possible, as used in molecular epidemiology. Recent interest in biological weapons has heightened advances in bacterial identification, but many of these methods are still not sufficiently sensitive to detect the low concentrations of pathogens considered important in drinking-waters. Hence, there is still a research need to develop routine approaches for where these organisms concentrate — e.g., biofilm-based detection methods or improved concentration methods for large-volume water samples. Overall, to broaden out knowledge of the heterotrophs of concern, sound collaboration between medical microbiologists and physicians is necessary to clarify the significance of unidentified heterotrophs or their

virulence factors in aquatic environments, among what appears to be an increasingly complex story (Feil and Spratt 2001).

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