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The Development of Gene Probes for Coliforms and Other Micro-Organisms (WMI 9018)

Final Report to the Department of the Environment
September 1988 to June 1993

DoE 3426/1
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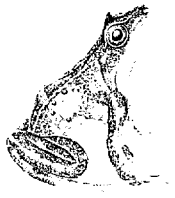

The Development of Gene Probes for Coliforms and Other Micro-Organisms (WMI 9018)

Final Report to the Department of the Environment
September 1988 to June 1993

Report No: DoE 3426/1

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SUMMARY

Gene probe is the term used to describe a novel molecular biological technique which detects pieces of genetic material specific to a species or group of organisms. It has been proposed that gene probes should allow the detection of specific microorganisms with greater speed and certainty than traditional analytical methods.

A major problem in drinking water microbiology is that current methods for the detection of the indicators of microbiological quality (total coliforms and *Escherichia coli*) take more than 24 h. As a consequence, monitoring can only provide retrospective information about quality.

There has been considerable speculation about how gene probes could revolutionise bacterial detection. In particular, it has been suggested that the time required for analysis of coliforms could be reduced to less than 6 h. This would be of great operational value in the control of drinking water quality.

For this reason the potential value of gene probe techniques in detecting bacteria in drinking water samples needed investigation. The Department of the Environment placed a contract with WRc to assess the potential use of gene probes and where necessary carry out further development specifically for the microbiological analysis of water. This research contract was steered by and carried out under the supervision of the Drinking Water Inspectorate.

This contract was to provide an independent and realistic assessment of the future role of these techniques to monitoring the microbiological quality of drinking water. Two aspects have been investigated:

1. The application of gene probe techniques to the enumeration of total coliform organisms and *E. coli* in drinking water.

2. The application of gene probe techniques to the detection of enteroviruses in drinking water.

The strategy adopted for enumeration of bacteria in drinking waters was to implement the gene probe methods into the existing traditional procedure of membrane filtration. The method appears to work although a robust method, which is significantly more rapid than the traditional method, has not been achieved because of limits in the currently available technologies for visualisation of the gene probes and their detection sensitivities. However, further developments should bring improvements within a few years.

A fundamental step in the detection of viruses by gene probes is the amplification of viral genetic material extracted from the water samples. The best currently available technique called PCR (polymerase chain reaction) was not found to have reached an adequate stage of development for the viruses of health significance in drinking water. However, it was found

that PCR could be applied to the detection of nucleic acid material from viruses of interest in other areas of public health.

This is a rapidly developing area of microbiological rapid methods. Whereas the contract has not provided complete robust methods which are directly applicable to the water industry, it has provided a unique insight into shortcomings of current technology and an indication of the immediate potential value of gene probe techniques. The information gained from the studies will also be extremely valuable to others interested in progressing this application of gene probe techniques.

EXECUTIVE SUMMARY

This contract from September 1988 to June 1993 was funded by the Department of the Environment (reference PECD 7/7/302) and was carried out under the supervision of the Drinking Water Inspectorate.

OBJECTIVES

The initial objectives were to develop and test gene probes for rapid (less than 6 h) detection of *Escherichia coli* and total coliforms for use in routine water laboratories. The probes were to be evaluated under a range of operational conditions and modified, where appropriate, to produce a robust technique. The project was extended with the objectives of applying gene probe methods to the detection of waterborne viruses of health significance in drinking water.

BACKGROUND

Advances in molecular biology and DNA biotechnology have laid the foundations to develop gene probe methods for detection and enumeration of microorganisms of health significance in drinking water. Gene probes may be designed to detect the genetic material (deoxyribonucleic acid, which is abbreviated to DNA, or ribonucleic acid, which is abbreviated to RNA) from any desired species of microorganism, e.g. *E. coli*, rotavirus, or poliovirus. The major advantage of gene probes is their potential for absolute specificity in the species of microorganism identified. This not only reduces the numbers of false negatives and false positives but also permits both the detection and the confirmation to be performed in a single step. Integration of gene probe technology into techniques for enumerating bacteria or detecting viruses in water samples thus offers the potential for more specific and more rapid methods.

STRATEGY

Design and development of DNA probes for total coliforms and *E. coli* were contracted to University of Leicester, which was a centre of excellence. Viral PCR primer sequences were obtained from University of Warwick.

Any method for detection of microorganisms in drinking water samples must be sensitive enough to detect down to a single viable organism (coliform or *E. coli*) in a 100 ml volume of water. A major problem in implementing gene probe technology to detecting microorganisms in water samples is the low sensitivity of detection and some form of amplification of bacterial or viral genetic material is necessary. The polymerase chain reaction (PCR) was used for amplification of viral genetic material. For bacteria (total coliforms and *E. coli*) amplification was performed by culturing after membrane filtration of the water sample. This offered two advantages over a PCR method. First, only those

bacteria which were culturable and thus alive would be detected. Second, total coliforms and *E. coli* could be enumerated by counting the numbers of labelled colonies.

Traditionally, radioactive labels are used for detecting DNA probes. These require special safety precautions and non-radioactive labelling systems were assessed for use with gene probe methods in routine water laboratories.

DETECTION OF TOTAL COLIFORMS AND *E. COLI*

The *lac* operon was identified by the external subcontractor as a gene probe specific for total coliform bacteria. *E. coli* specific oligonucleotide probes were developed for 16S ribosomal RNA (rRNA) sequences. One problem encountered in implementing gene probe methods to the detection of bacterial colonies was the elimination of non-specific binding by components of the non-radioactive gene probe labelling systems to colony debris. This was achieved by treating the lysed colonies with proteinase K prior to hybridisation with the probe. Using non-radioactively labelled *lac* gene probe, total coliform colonies were confirmed on membrane filters within 42 h after membrane filtration. This offered some improvement in detection time over the standard UK membrane filtration method for total coliforms which requires up to 72 h for completion including confirmation. Efficiency in detection of *E. coli* colonies was compromised in that an incorrect probe sequence was supplied by the external subcontractor. Essentially, however, the method to detect *E. coli* colonies with a non-radioactive 16S rRNA probe appears to have been successful when applied to the large colonies grown up from neat cultures. *E. coli* colonies generated stronger signals when hybridised with the probe than those of *Aeromonas hydrophila*, while *Citrobacter freundii* colonies were not detected. However, the signal intensity from the *E. coli* colonies was weak and colonies were not detectable by the gene probe on membrane filters after direct filtration and culturing (18 h) from environmental water samples.

DETECTION OF WATERBORNE VIRUSES

For poliovirus and rotavirus, oligonucleotide primers were obtained for amplification of specific sequences within the viral genetic material by PCR. These waterborne viruses, as indeed do most viruses of health significance in drinking water, contain RNA genomes which must first be converted to DNA before amplification by the PCR method. Commercially available kits for performing PCR from RNA starting material were found to experience problems on converting the RNA to DNA. Furthermore the process of PCR appeared to be highly sensitive to inhibitors present in the water. Indeed molecular biology grade water was required for efficient functioning of the RNA PCR kits and rotavirus RNA extracts were found to inhibit PCR amplification of a DNA positive control. Detection of RNA from poliovirus and rotavirus cultures was not successful at WRc, although amplification and detection of DNA from adenovirus was achieved by PCR.

PRACTICAL IMPLICATIONS

The use of gene probes for the confirmation of bacterial colonies after membrane filtration is applicable to the water industry. However, production of a robust and reliable method, which is significantly more rapid than standard UK methods and which is easily implemented in routine laboratories does not appear to be achievable at present.

The use of PCR as a routine detection method for viruses in water laboratories does not appear to be feasible at present. Inhibitors of PCR appear to be a major problem, and the viral genomic material would have to be highly purified to remove the inhibitors.

1. INTRODUCTION

This contract from September 1988 to June 1993 was funded by the Department of the Environment (reference PECD 7/7/302) and was carried out under the supervision of the Drinking Water Inspectorate.

In the UK, legislation (Water, England and Wales, No. 1147, The Water Supply (Water Quality) Regulations 1989; No 1837, Amendment 1991) requires the water supply undertakings to supply wholesome water. In addition each water supply undertaking is obliged to monitor water taken from consumers' taps (fixed point and random) for compliance with these regulations. The bacteriological quality of the water is monitored by measuring concentrations of total coliforms, thermotolerant coliforms, faecal streptococci, sulphite-reducing clostridia and colony counts.

Bacteria belonging to the group of organisms called total coliforms serve as primary indicators of faecal pollution in drinking water. Recently the definition of total coliform organisms has changed. Under the old definition (DoE *et al.* 1983), coliforms were Gram-negative, non-sporing rod-shaped bacteria, capable of aerobic and facultatively anaerobic growth in the presence of bile salts or other surface active agents with similar growth-inhibiting properties, which are able to ferment lactose within 48 hours at 37 °C with the production of acid and gas. The new definition (DoE 1990) defines total coliforms as members of a genus or species within the family *Enterobacteriaceae*, capable of growth at 37 °C, and normally possessing the enzyme β-galactosidase. This enzyme is the first in the metabolic pathway of lactose utilisation. Of particular health significance is the coliform called *Escherichia coli* which is undoubtedly faecal in origin. *E. coli* refers to thermotolerant coliform organisms which ferment lactose at 44 °C with the production of acid and gas within 24 hours, and which also form indole from tryptophan.

Under the Water Supply (Water Quality) Regulations 1989 (Amendment 1991) *E. coli* should not be detectable in any 100 ml volume drinking water sample. Furthermore, no sample of 100 ml should contain any total coliform organisms or faecal streptococci. In practise, however, such a total coliform standard is not always attainable and a 95% compliance is enforced for total coliforms. This means that for any zone, coliform organisms should not occur in more than 5% of routine samples, provided that at least 50 samples have been examined at regular intervals throughout the year.

Methods of enumeration of *E. coli* and total coliforms currently accepted in the UK are presented in Report 71 (DoE *et al.* 1983) and are based on conventional techniques requiring two to four days for completion, including confirmation. In the membrane filtration method, 100 ml volume water samples are filtered and colonies cultured by incubation on membrane lauryl sulphate broth (DoE *et al.* 1983). After an 18 h culture, presumptive total coliforms or *E. coli* are enumerated on the basis of the number of colonies. Individual colonies are then confirmed, requiring a further 24-48 h.

The advances made in molecular biology over the last two decades have raised the possibility of developing new techniques, which are based on gene probe technology, for the detection and identification of a particular species of virus or bacterium. Gene probes

may be designed to detect the genetic material, i.e. deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), from any desired species of microorganism, e.g. *E. coli*, rotavirus, or poliovirus. Furthermore, a single gene probe may be developed to detect groups of organisms e.g. bacteria belonging to the group of organisms called total coliforms. The major advantage of gene probes is their potential for absolute specificity in the species of microorganism identified. This not only reduces the numbers of false negatives and false positives but also permits both the detection and the confirmation to be performed in a single step. Integration of gene probe technology into techniques for enumerating bacteria in water samples thus offers the potential for more specific and more rapid methods.

Conventional methods for detecting viruses in water samples are based on concentration of the virus and detection through the cytopathic effects of culturing the virus in animal cell lines. For the three serotypes of poliovirus, the coxsackievirus group B and echovirus, this method is routine. However, some viruses such as rotaviruses are culturable in animal cell lines but are not cytopathic and thus alternative methods of detection are required. Gene probes offer the potential for not only detecting the presence of viral nucleic acids in such cultures but also specifically identifying particular species and strains. Furthermore, other waterborne viruses, such as Norwalk virus and hepatitis A virus are not culturable and currently very difficult to detect in environmental samples. It is hoped that gene probes may provide a method for detecting and identifying such agents in water samples.

The major problem with gene probes is their lack of sensitivity and single microorganisms cannot be detected without amplification of some form. For bacteria this may be performed on membrane filters on which individual bacteria multiply into colonies. Detection of viral nucleic acid would be much more difficult but for the advent of the polymerase chain reaction (PCR) in which selected sequences may be amplified over a million fold.

In this report the details of the methods and results are presented in Appendices. Traditionally gene probes have relied on radioactive methods for their detection. However, these require safety precautions and Appendix A presents the various non-radioactive detection methods implemented for using gene probes for detection of bacteria in water samples. Appendix B details the methods for detection of bacterial colonies on membrane filters. These include methods for colony lysis, hybridisation of the probes and the control of hybridisation stringency by washing. In Appendices C and D, the results of experiments to detect total coliforms and *E. coli*, respectively, on membrane filters are presented. Appendix E reports the problems encountered on using RNA-PCR kits to detect poliovirus or rotavirus RNA.

The major principles and conclusions of the work are presented in the following part of the report. The objectives of the contract are stated in Section 2. Section 3 introduces the concepts of gene probes and Section 4 summarises the principles of gene probe design and development, including labelling with non-radioactive detection systems. Section 5 outlines the strategy and results for the application of gene probes for enumerating total coliforms and *E. coli* in water samples. Attempts to use gene probes to detect waterborne

viruses by PCR are reported in Section 6. The conclusions are presented in Section 7 and recommendations made in Section 8.

Like many specialised fields, gene probe technology has acquired its own terminology. To assist readers who are unfamiliar with this field a glossary is included in Section 1.1.

1.1 Glossary

DNA - deoxyribonucleic acid comprises the genetic material (chromosomes) of bacteria, animal and plant cells.

RNA - ribonucleic acid comprises genetic material of certain viruses and plays important roles in functioning of bacterial, plant and animal cells.

Gene Probe - DNA molecule of defined base sequence which binds (by hybridisation) to a specific region (target site) of DNA from a bacterium or virus and which is labelled to allow its detection.

PCR primer - short DNA molecule from which PCR is initiated.

Hybridisation - binding of the DNA probe to its specific target site on the DNA (or RNA) of a bacterium or virus.

Polymerase chain reaction (PCR) - method to amplify (over a million fold) a specific sequence of DNA or RNA as defined by two PCR primers.

Lac operon - part of coliform DNA coding for proteins which allow utilisation of lactose.

16S rRNA - name for an RNA component of ribosomes in bacterial cells.

Oligonucleotide - short DNA molecule.

2. OBJECTIVES AND PROGRAMME OF WORK

The project commenced in September 1988.

The objectives were:

1. To develop and test a gene probe for rapid (less than 6 h) detection of *E. coli* suitable for use in routine water laboratories.

2. To develop a gene probe (or gene probes) for the detection of those species of bacteria collectively known as total coliforms.

3. To evaluate the developed probe or probes under a wide range of operational conditions, and where appropriate modify the form of the probe to produce a robust technique.

From July 1991 the project was extended with the objectives of applying gene probe methods for the detection of viruses of health significance in drinking water. In the programme of work it was proposed to implement the method with adenovirus and poliovirus. The method for poliovirus should be applicable to hepatitis A virus and Norwalk virus by changing the PCR primers. A method was also to be developed for the detection of rotavirus.

3. WHAT ARE GENE PROBES AND HOW DO THEY WORK?

A gene probe is a sequence of deoxyribonucleic acid (DNA). DNA is the material which specifies the genetic code and comprises the chromosomes of each and every cell. The code is specified in the sequence of chemicals called bases. There are four bases; adenine (A), guanine (G), cytosine (C) and thymine (T). A chromosome may contain over a million of these bases. The order of bases within a chromosome differs from species to species. Certain regions of bases within a chromosome code for proteins, which perform specific functions within the cell. These regions are called genes. The units which link together to form a DNA molecule and which hold the bases are called nucleotides. Each nucleotide contains one base.

A DNA probe may be as short as 15 nucleotides, i.e. 15 bases. These short probes are called oligonucleotide probes (oligo = few). Other DNA probes may be as long as 100 000 nucleotides. These are called gene probes. The sequence (i.e. order and composition of bases) of a gene probe is critical for its function. A gene probe with a specific sequence of bases will bind to an exactly complementary sequence by a process called hybridisation. Hybridisation is mediated by complementary base-pairing. Adenine always base-pairs with thymine and thymine always base-pairs with adenine. Guanine always base-pairs with cytosine and cytosine always base-pairs with guanine. Thus a gene probe may be designed to bind to a particular sequence of DNA from a desired organism by making its sequence exactly complementary. The DNA sequence from the organism which is to be detected by the gene probe is called the target site and must be determined by a process called DNA sequencing before gene probes may be designed to detect it.

To use a gene probe to detect a particular microorganism, the DNA from the organism of interest is immobilised onto the surface of a nitrocellulose filter. The filter is immersed in a solution (the hybridisation buffer) containing the gene probe. The gene probe, if complementary to a sequence on the organism's DNA, hybridises to that sequence (the target sequence) by complementary base pairing. The hybridisation conditions (i.e. nature of the hybridisation buffer and temperature) are critical. Indeed there is potential for oligonucleotide probes which differ by a few bases compared to the exact complementary sequence to bind to "false" target sites under certain conditions. In these conditions the stringency of hybridisation is said to be low. However, by changing the temperature, urea concentration or ionic strength of the hybridisation buffer, it is possible to increase the stringency of hybridisation such that exact matching between all bases on the probe and target sequence must be achieved for successful hybridisation of the probe.

Once bound to its target sites on the organism's DNA which is fixed to the nitrocellulose filter, the gene probe must be detected. Thus before hybridisation the gene probe is labelled with a marker to enable detection. Traditionally, the DNA probe is synthesised with radioactive nucleotides (usually with ^{32}P replacing ^{31}P in the phosphate group). However, considerable research effort is currently underway in companies such as Amersham and Boehringer to produce kits for labelling and detecting gene probes with non-radioactive markers.

4. DEVELOPMENT OF DNA PROBES AND PCR PRIMERS FOR DETECTION OF BACTERIA AND VIRUSES OF HEALTH SIGNIFICANCE TO THE DRINKING WATER INSPECTORATE

Development of DNA probes comprises of two stages:

- identification of the nucleotide sequence
- labelling the DNA probe with a non-radioactive marker

In this section the identification of sequences of DNA probes for total coliform and *E. coli* bacteria is discussed. Details of development of probes for total coliforms and *E. coli* are presented in Appendices C and D, respectively. PCR primer sequences for viruses of health significance in drinking water are reported in Appendix E.

The non-radioactive labelling and detection kits are introduced and the problems experienced in implementing these kits for detection of bacterial colonies are reported.

4.1 Probe sequences

The species specificity of a DNA probe is determined by its nucleotide sequence. Gene probe design requires identification of sequences which are unique to the desired target organism. Development of species-specific gene probes and PCR primers for bacteria and viruses of health significance in drinking water was not performed at WRc.

Design of probes for *E. coli* and total coliforms was subcontracted to University of Leicester. The work was performed there during 1989 and 1990. University of Leicester identified the *lac* operon as a potential target site for a total coliform specific gene probe (Appendix C.1). DNA sequences containing all or part of the *lac* operon would thus serve as total coliform-specific gene probes. Design of *E. coli* specific probes was directed at the 16S ribosomal RNA (rRNA) molecules which comprise ribosomes. Ribosomes are cellular components and may number up to 10 000 per cell. Cells from all bacteria contain 16S rRNA molecules. Certain regions of these molecules are known to vary between different organisms. By sequencing the 16S rRNA molecules from a variety of bacteria, University of Leicester were able to identify four such sequences unique to *E. coli*. Oligonucleotide probes for these sequences were synthesised and are referred to as 16S rRNA probes. The sequences for these probes are presented in Appendix D.1. The probe which was named Ec1000 was judged by University of Leicester to be most appropriate. Unfortunately, the nucleotide sequence which University of Leicester presented to WRc for the probe Ec1000 was later found by WRc to have two bases missing. This may account to some extent for the low sensitivity observed in labelling *E. coli* colonies with Ec1000. This is discussed in Appendix D.2.

Design of gene probes or PCR primers for viruses is a less formidable task than for bacteria for two reasons:

1. virus genomes are much smaller than those from bacteria.

2. complete nucleotide sequences for DNA or RNA from many viruses of health significance to the water industry are available in the literature. Thus, sequences for poliovirus serotypes, rotaviruses, adenoviruses, hepatitis A virus, Norwalk virus and human immunodeficiency virus are available. These may be used for obtaining sequences for oligonucleotide probes.

The design and specification of nucleotide sequences for viral gene probes and PCR primers do not require the practical input necessary for the *E. coli* probes described above. PCR primer sequences for two viruses of waterborne health significance have been obtained from Professor M McCrae at University of Warwick. These were rotavirus and poliovirus. Primer sequences for these viruses and also for adenovirus are presented in Appendix E.2.

4.2 Labelling and detection of gene probes with non-radioactive kits

Three types of non-radioactive labelling kits were assessed for detection of *E. coli* and total coliform colonies using 16S rRNA oligonucleotide probes and the *lac* operon probe, respectively. These kits are listed:

- Blu-GENE biotin detection kit.

- Amersham enhanced chemiluminescent (ECL) labelling and detection kits based on the light-producing enzyme, horse-radish peroxidase (HRP).

- Boehringer digoxigenin (DIG) DNA labelling and detection kit.

The design and principles of these kits are described in Appendix A.

The major problem encountered with the biotin and ECL kits arose from non-specific binding of parts of the detection system to components of the remains of the bacterial colonies on the membrane filters.

The Blu-GENE detection kit was used by University of Leicester for detecting *E. coli* colonies with biotin-labelled Ec1000 probe and total coliform colonies with biotin-labelled *lac* operon probe. Unfortunately, the kit was designed for detecting pure DNA extracts and not for the application of detecting colonies. The presence of biotin endogenous to the bacterial cells within the colonies resulted in the Blu-GENE detection kits labelling colonies from a variety of species such that species specificity from target DNA/DNA probe hybridisation was eliminated (Appendix D.3).

The Amersham ECL labelling and detection kits were also prone to generating false positives in the application of colony hybridisation. Indeed, HRP from the 5'-oligonucleotide labelling and detection kit (Appendix A.2) was found to label bacterial colonies even when it had no oligonucleotide probe attached. This problem was overcome by using the Amersham 3'-oligolabelling kit instead and treating the filters with proteinase K prior to hybridisation (Riley and Caffrey 1990).

5. ENUMERATION OF TOTAL COLIFORMS AND *E. COLI* IN WATER SAMPLES USING GENE PROBES

In this section the strategy for enumerating total coliform and *E. coli* colonies using gene probe methods is described. The results obtained are discussed.

5.1 Strategy for enumeration of total coliforms and *E. coli* in water samples using gene probes

The strategy adopted for using gene probes to enumerate total coliforms or *E. coli* in water samples was to implement the gene probe as a confirmation method for presumptive colonies cultured on membrane filters. The standard UK membrane filtration method (DoE *et al.* 1983) involves a 4 h incubation at 30 °C followed by 14 h at 37 °C for total coliforms or at 44 °C for *E. coli*. It was anticipated that detection of smaller colonies could be achieved using the gene probe detection method such that the incubation period could be reduced to less than 18 h. For the DNA probe to bind to its target sites within the bacterial cells, it was necessary to lyse the cells within the colonies. For DNA target sites e.g. *lac* operon or 16S rRNA genes, this was performed using 0.5 M NaOH (Riley and Caffrey 1990). However, for RNA target sites, e.g. the 16S rRNA molecules within the ribosomes, the anionic detergent sodium dodecyl sulphate (SDS) was used (Ivanov and Gigova 1986). Colony lysis methods are described in Appendix B.1. The genetic material was then fixed to the surface of the nitrocellulose filters by baking after which hybridisation of the labelled gene probe was performed (Appendix B.2). Stringency of hybridisation was controlled by varying the temperature, urea concentration or ionic strength during the washing steps (Appendix B.2). It was hoped that the gene probe membrane filtration method would allow enumeration of confirmed organisms within 24 h.

A major problem with using gene probes to detect DNA from a target organism is the low sensitivity. A single gene probe labelled with HRP, DIG or a radioactive atom (e.g. ^{32}P) cannot be detected. Only multiple copies of a gene probe may be detected. Since each gene probe requires one copy of a target site to bind to, the application of using gene probes for enumerating bacteria in water samples is dependent on the presence of multiple copies of the target site DNA. Genes such as the *lac* operon are present at only one copy per cell. Thus detection of coliforms using the *lac* gene probe requires amplification of the genomic material. Culturing individual bacteria into colonies on membrane filters provides the necessary amplification of genetic material for detection by the gene probe. Bej *et al.* (1990) have used PCR to amplify the *lac* operon of coliform bacteria and this method may be used to detect the presence of coliforms in water. However, the PCR method does not allow enumeration of bacteria giving only a presence/absence result. Furthermore PCR would not discriminate between viable and non-viable coliforms and its application to analysis of chlorine-treated drinking waters would appear to be limited for this reason.

University of Leicester identified the 16S rRNA sequences as potential target sites for *E. coli* gene probes not only because the variation between species offered an *E. coli*

specific probe but also because the 16S rRNA sequences may be present at approximately 10 000 copies per cell. Thus, each *E. coli* cell could potentially bind 10 000 copies of a 16S rRNA oligonucleotide probe. This would improve sensitivity and facilitate detection of *E. coli* cells. University of Leicester were optimistic that the large number of target sites for 16S rRNA oligonucleotide probes would greatly reduce the time required for culturing colonies before detection could be achieved with the probe.

5.2 Detection of total coliform colonies on membrane filters using the *lac* gene probe

Bacteria belonging to the group of organisms called total coliforms serve as primary indicators of faecal pollution in drinking water. Recently the definition of total coliform organisms has changed. Under the old definition (DoE *et al.* 1983), coliforms were Gram-negative, non-sporing rod-shaped bacteria, capable of aerobic and facultatively anaerobic growth in the presence of bile salts or other surface active agents with similar growth-inhibiting properties, which are able to ferment lactose within 48 hours at 37 °C with the production of acid and gas. The new definition (DoE 1990) defines total coliforms as members of a genus or species within the family *Enterobacteriaceae*, capable of growth at 37 °C, and normally possessing the enzyme β-galactosidase. This enzyme is the first in the metabolic pathway of lactose utilisation and is coded for in the bacterial chromosome by the *lac* operon. Using the *lac* gene probe which selectively targets the *lac* operon is thus a natural way to specifically detect total coliform bacteria.

Methods and results from experiments to enumerate total coliforms in water samples using the *lac* gene probe are detailed in Appendix C.

The *lac* gene probe was found to exhibit a high degree of specificity in detecting colonies from coliform bacteria, i.e. those which produced acid and gas from membrane lauryl sulphate. However, there were three species of non-coliform *Enterobacteriaceae* which only produced acid from membrane lauryl sulphate and which were also detected by the *lac* operon gene probe.

Using ^{32}P-labelled *lac* gene probe, University of Leicester was able to detect total coliform colonies on membrane filters within 35 h of filtration. The biotin-labelled probe required only 16 h, although binding was non-specific and thus the method invalid (Section 4.2). Using the non-radioactive HRP-labelled *lac* gene probe, it was possible to detect confirmed total coliform colonies 42 h after filtration of the water sample. Proteinase K treatment of the colonies after NaOH lysis and prior to hybridisation (Appendix B.1.2) was essential to eliminate non-specific binding of the HRP-labelled probe to colony remains. This method offers some improvement in detection time compared to the standard UK method which requires up to 72 h (DoE *et al.* 1983).

5.3 Detection of *E. coli* colonies on membrane filters using the 16S rRNA probes

Methods and results from experiments to enumerate *E. coli* in water samples are presented in Appendix D.

Detection of *E. coli* colonies using the 16S rRNA probe Ec1000, labelled with horse-radish peroxidase (HRP) was set back by two problems.

First, the HRP protein was found to be sticky and bound strongly to debris on the remains of the colonies of the membrane filters. The 5'-oligolabelling kit was particularly prone to non-specific binding to colonies by HRP. The problem was overcome by using the Amersham 3'-oligolabelling kit and treating the lysed colonies with protienase K to remove colony debris prior to hybridisation (Appendix D.4).

Second, University of Leicester, who were subcontracted to develop *E. coli* specific 16S rRNA probes, entered the published sequence (Ehresmann *et al.* 1977) incorrectly into the computer. This error resulted in Ec1000 being identified as an *E. coli* specific probe. The effects of this error (detailed in Appendix D.2) are two-fold:

- Ec1000 may not be specific for *E. coli* DNA alone.
- binding potential for the probe used by WRc throughout most of this contract may be impaired.

As discussed in Appendix D.2 the error may not entirely eliminate binding of the probe used by WRc. Indeed, the probe sequence used bound strongly to commercially available *E. coli* DNA extract (Appendix D.3) with no binding to DNA from *Micrococcus lysodeikticus* or from M13 virus. However, a slight cross reaction was observed with DNA from *Clostridium perfringens*. Increasing the stringency of hybridisation with urea (Appendix B.2) did not significantly reduce Ec1000 binding to *E. coli* DNA, which is an encouraging result. Furthermore, the cross-reaction of Ec1000 with *Clostridium perfringens* DNA could be eliminated by increasing the temperatures of the washing steps from 42 to 55 °C. These experiments provided useful information on controlling the species specificity of oligonucleotide probes by the stringency of hybridisation and suggested that experimental conditions will need to be strictly controlled if gene probe methods are adopted in routine analysis in water laboratories.

The method to detect *E. coli* colonies with a non-radioactive 16S rRNA probe appears to have been successful when applied to the large colonies grown up from neat cultures. *E. coli* colonies generated stronger signals when hybridised with HRP-labelled Ec1000 probe than those of *Aeromonas hydrophila*, while *Citrobacter freundii* colonies were not detected. However, the signal intensity from the *E. coli* colonies was weak and colonies were not detectable by the probe Ec1000 on membrane filters after direct filtration and culturing (18 h) from environmental water samples.

6. DETECTION OF VIRUSES IN WATER SAMPLES USING PCR

Methods and results of experiments to amplify and detect genomic RNA from poliovirus and rotavirus are reported in Appendix E. In addition, the molecular biology of these viruses is introduced in Appendix E.1.

6.1 Strategy for detection of viruses in water samples using gene probes

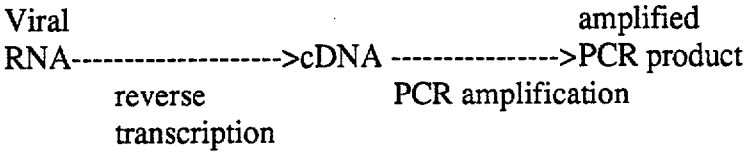
There are several potential problems in developing a gene probe method for detecting and identifying viral genetic material in water samples. These are:

- extracting and concentrating the viral genetic material from water samples perhaps as large as 10 litres into volumes small enough for performing gene probe techniques (perhaps 5-100 µl).
- amplifying the potentially low levels of viral genetic material into quantities that are large enough to be detected by gene probe methods.
- discriminating between infectious and non-infectious virions.

Initial experiments were therefore performed to detect viral nucleic acid in neat cultures before attempting to extract and detect viral nucleic from water samples. Viral nucleic acid was extracted and concentrated (Appendix E.3.1). Amplification of specific parts of the viral nucleic acid was performed by the polymerase chain reaction (PCR).

The region of nucleic acid sequence to be amplified by PCR is determined by two primers. One primer is called the downstream primer, the other the upstream primer. Each primer is a short oligonucleotide of defined sequence which hybridises to its exactly complementary sequence at the downstream or upstream end of the region of DNA to be amplified. Selection of the sequence which is to be amplified depends on hybridisation of the two primers to the respective ends of that sequence. Thus, by altering the nucleotide sequence, primers may be developed which specifically amplify a sequence of poliovirus genetic material at the exclusion of all other sequences. Amplification is performed by extension of the primers by a DNA polymerase enzyme. Each round of amplification theoretically doubles the amount of the specific DNA sequence and is performed by regulating the temperature using a thermal cycler (PCR machine).

The viruses of major health significance to the water industry (poliovirus, hepatitis A virus, Norwalk virus and rotavirus) are comprised of an RNA genome. Conventional PCR amplifies DNA and thus a method which converts RNA sequences into the corresponding double-stranded complementary DNA (cDNA) is required. This is provided by the technique of RNA PCR, in which the enzyme reverse transcriptase first copies the viral RNA genome into complementary cDNA. The double-stranded cDNA product is then used as a substrate for conventional PCR.



Amplified DNA products are detected either by agarose gel electrophoresis or by fixing to a nitrocellulose filter and hybridising with a gene probe.

Two commercial RNA PCR kits for PCR amplification from RNA starting material were assessed. These were the rTth RNA PCR kit and the retrovirus reverse transcriptase (RTase) RNA PCR kits. These kits obtained from Perkin Elmer Cetus are described in Appendix E.3.2. The strategy for detection of poliovirus genomic RNA was to amplify a selected sequence of 530 basepairs using the two PCR primers, PG1 and PG2 (Appendix Table E.1) with the rTth RNA PCR kit. A similar approach was adopted for detecting rotavirus RNA using PCR primers, G1 and G2 (Appendix Table E.2) and the RTase RNA PCR kit. As a positive control for the PCR amplification, adenovirus DNA was amplified using the two PCR primers, A1 and A2 (Appendix Table E.3) and AmpliTaq polymerase from the RTase RNA PCR kit.

The method for poliovirus should be applicable to hepatitis A virus and Norwalk virus by changing the PCR primers.

6.2 Detection of viruses by PCR

Initial attempts to amplify positive control RNA provided with the rTth RNA PCR kits were unsuccessful. However, after consultation with Perkin Elmer Cetus it was found that by using molecular biology grade water (Sigma) positive control RNA could be amplified. This indicates that high purity conditions are required for successful PCR amplification of RNA genomes. Amplification of positive control RNA with the RTase RNA PCR kit was successful approximately 50% of the time. However, all attempts to amplify poliovirus RNA with the rTth RNA-PCR kits and to amplify rotavirus RNA with the RTase RNA-PCR kit were unsuccessful.

PCR amplification of a 411 base pair sequence from commercially available adenovirus-2 DNA using the AmpliTaq DNA polymerase provided with the RTase RNA PCR kits was successful and furthermore results were reproducible. However, on addition of rotavirus RNA extract, PCR amplification of adenovirus DNA was inhibited suggesting that the rotavirus RNA extract contained an inhibitor of PCR.

7. CONCLUSIONS

7.1 Total coliforms and *E. coli*

The approach adopted to enumerate total coliform and *E. coli* bacteria in water samples was to use gene probes to identify and confirm the colonies cultured on membrane filters. This offered two advantages over a PCR method:

- bacteria are enumerated.
- only bacteria which are able to multiple into colonies and which are therefore alive are detected.

The potential advantages offered over traditional membrane filtration were that:

- all *E. coli* or total coliform colonies would be confirmed in one step.
- detection of colonies would be achievable more quickly so reducing the overall detection time.

Detection of total coliform and *E. coli* colonies with non-radioactive DNA probes appears to have been successful. For total coliforms a gene probe which targeted the *lac* operon was used. *E. coli* were detected with an oligonucleotide probe which targeted a sequence on the 16S rRNA. Species specificity of the *E. coli* probe has not been evaluated due to the finding of an error in the sequence provided by the subcontractor. The low sensitivity of gene probe methods, however, has prevented significant improvements in detection time over standard membrane filtration methods. Development of methods which enumerate culturable bacteria in less than 6 h does not appear to a realistic target at present.

Problems were experienced in implementation of non-radioacitve labelling methods. In particular, colonies were prone to non-specific binding and labelling by parts of the probe detection system. However, choice of kit and removal of colony debris by enzymatic digestion prior to hybridisation with the probe eliminated this problem. Protocol conditions may be critical to ensure desired bacterial specificity of probe.

7.2 Waterborne viruses

The approach adopted at WRc was to use PCR to amplify viral genetic material for detection by gel electrophoresis. This would provide a presence/absence test specific for particular viruses of health significance in drinking water.

The viruses of major health significance in drinking water include rotaviruses, poliovirus, hepatitis A virus and Norwalk virus. These viruses contain RNA genomes. PCR amplification of RNA genomes was performed in two stages; reverse transcription of the RNA into DNA, which was then amplified by conventional PCR. All attempts to amplify

RNA from poliovirus and rotavirus were unsuccessful. However, amplification of adenovirus DNA was successful, suggesting that conversion of poliovirus or rotavirus RNA into DNA by reverse transcription was the step that was failing. However, inhibition of PCR by certain components within the extracted RNA was also found to be a problem. Thus, rotavirus RNA extract inhibited PCR amplification of adenovirus DNA. Furthermore problems in amplification of positive control RNA provided with the Perkin Elmer PCR kits were overcome by using molecular biology grade water suggesting that RNA extracts must very highly purified to overcome inhibition of PCR. Purification of genomic RNA by ethanol precipitation may not in itself be sufficient to remove inhibitors of PCR amplification. Similarly, Alexander and Morris (1991) report that phenol extraction of nucleic acids does not remove inhibitors.

8. RECOMMENDATIONS

Production of a robust and reliable method for enumerating faecal indicator bacteria, which is significantly more rapid than standard UK methods and which is easily implemented in routine laboratories will not be achievable until problems with low sensitivity of gene probe detection are overcome. It is recommended that further work is required to:

1. develop methods for fixing more genetic material from individual bacterial colonies onto the membrane filter;

2. enhance the signal from the gene probe labelling system. This requires development of more sensitive non-radioactive gene probe labelling and detection kits by companies such as Amersham International and Boehringer Mannheim.

Inhibitors of PCR appear to be a major problem in the detection of viruses in water samples by gene probes and high purification of the viral genetic material is required. The highly labile nature of the genetic material (RNA) from viruses of heath significance in drinking water and the need to detect small amounts in large water volumes makes gene probe methods to detect viruses in water infeasible for application in routine laboratories. However, gene probe PCR methods for viruses would find an application in clinical and public health laboratories.

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APPENDIX A PROBE DETECTION BY NON-RADIOACTIVE LABELLING KITS

Traditionally gene probes have been labelled by ^{32}P. The high energy radiation emitted by the ^{32}P nucleus requires special safety precautions when handling the probes. To make implementation of gene probe methods for routine enumeration of bacteria in water samples more acceptable to the water industry, WRc used a variety of non-radioactive labelling methods for detecting gene probes. Three gene probe labelling methods, commercially available as kits and used in this project are described below.

A.1 Biotin labelled probes

Both biotin-labelled *lac* and biotin-labelled 16S rRNA probes were used by University of Leicester. Bethesda Research Laboratories market the BluGENE kit for colorimetric detection of biotin-labelled gene probes. The kit provides a streptavidin-alkaline phosphatase conjugate, which is a streptavidin molecule linked to an enzyme of alkaline phosphatase. Streptavidin binds with very high affinity to the biotin labels on the gene probes. The presence of bound biotin-labelled gene probes, and hence the part of the filter where bacterial target DNA is, may be detected through the colorimetric reaction catalysed by the enzyme alkaline phosphatase. Bacterial colonies appear as purple dots, the resolution of which may be increased by exposure of the filter to toluene.

A.2 Horse-radish peroxidase (HRP) labelled probes and enhanced chemiluminescence (ECL)

Amersham International have developed a series of gene probe detection kits based on the enzyme horse-radish peroxidase (HRP). HRP catalyses a light emitting reaction (enhanced chemiluminescence, ECL) which may be detected by photographic film. The kits provide a method to attach a molecule of HRP to the gene probe nucleic acid, producing an HRP-labelled gene probe. Two types of kits are available and both have been used in this project. These are now described.

1. The ECL oligonucleotide labelling kits are designed for labelling oligonucleotides comprised of between 10 and 50 nucleotides. These kits were used for labelling the 16S rRNA probes developed by University of Leicester for *E. coli*. Originally the kits provided an activated form of HRP which added covalently onto the 5'-end of the oligonucleotide. These 5'-oligolabelling kits required the oligonucleotide to be synthesised with a thiol linker at the 5' end. The thiol linker contained a protected sulphur atom which added chemically to the activated HRP thus joining the HRP to the probe. The 5'-oligolabelling kit has now been replaced by the 3'-oligolabelling kit in which the 3'-end of the oligonucleotide is modified with fluorescein-labelled nucleotides. HRP is not covalently linked to the gene probe itself, but is conjugated to an antibody which binds to the fluorescein molecules attached to the oligonucleotide.

2. The ECL gene probe labelling kits are designed for labelling much longer nucleotide sequences with HRP. Such sequences are typically longer than 1000 basepairs and are referred to as gene probes to distinguish them from the much shorter oligonucleotide probes. The HRP is not chemically activated to bind to thiol groups as in the 5'-oligonucleotide labelling kits (described above), but instead has been modified to in such a way as to give it a net positive charge. Positively charged HRP molecules bind electrostatically to negatively charged phosphate groups on the DNA probe. Glutaraldehyde is then added to cross-link the HRP to the DNA. Amersham report that HRP molecules bind to the DNA probe every 30 nucleotides and many HRP molecules may bind to a single *lac* gene probe molecule.

A.3 Digoxigenin (DIG) labelled probes

Boehringer Mannheim market the DIG DNA labelling and detection kit. This kit was chosen because it is recommended for the application of colony blotting (although it should be noted that the Amersham ECL kits were also recommended for this application and yet suffered from non-specific binding of HRP to colony debris).

As with the ECL kits, two versions of the DIG system are supplied; the gene probe kit for long nucleotide probes and the oligonucleotide kit for oligonucleotides. The gene probe kit was used to produce *lac* gene probes labelled with a steroid-like compound called digoxigenin. The digoxigenin-labelled *lac* probes, once hybridised to target DNA on the membrane filters are detected by the enzyme alkaline phosphatase which is conjugated to an anti-digoxigenin antibody. This antibody binds to digoxigenin molecules on the *lac* gene probe. The alkaline phosphatase catalyses conversion of a colourless substrate to a coloured product.

APPENDIX B METHODS FOR DETECTION OF *E. COLI* AND TOTAL COLIFORM COLONIES ON MEMBRANE FILTERS USING GENE PROBES

B.1 Culture and lysis of bacterial colonies on membrane filters

Circular filters (47 mm diameter) were cut out from Hybond-C super nitro-cellulose sheets (Amersham International). Water samples (10-100 ml volume) were filtered through these filters and incubated on membrane lauryl sulphate broth. A variety of incubation times and temperatures were assessed.

Several published protocols for lysis of cells comprising colonies and fixation of the cellular RNA (for 16S rRNA *E. coli* probes) or DNA to the filter were used. These are now described.

B.1.1 Alkaline lysis for detecting DNA target sites

Sodium hydroxide solution was used for lysing the cells comprising colonies to expose and denature the DNA for hybridisation. The method for colony lysis was adapted from that used by Mason and Williams (1988). Filters with bacterial colonies were placed on filter pads (3 min; room temperature) soaked in 1.5 M NaCl, 0.5 M NaOH. The alkali was neutralised by incubation (7 min) of the filters on pads soaked in 1.5 M NaCl, 1 M Tris-HCl (pH 7.5). The filter was then washed by placing on a pad soaked in 4x SET buffer (20x SET = 3 M NaCl, 20 mM EDTA, 0.4M Tris-HCl, pH 7.8) and finally baked at 75-80 °C (2 h).

B.1.2 Modified alkaline lysis method of Riley and Caffrey (1990) for detecting DNA target sites

The alkaline lysis method of Mason and Williams (1988) was found not to be appropriate for HRP-labelled gene probes (Appendices C.3.2 and D.4) or the DIG-DNA detection system since proteinaceous debris in the remains of the colony causes non-specific binding of the probes. To overcome this the colonies were treated with proteinase K as described by Riley and Caffrey (1990). Proteinase K is an enzyme which digests proteins.

The filters were placed on filter pads soaked in 1.5M NaCl, 0.5M NaOH and steamed in a pressure cooker (7 min) to lyse the colonies. The alkali was neutralised by two incubations (3 min) on filter pads soaked in 1.5M NaCl, 0.5M Tris-HCl, 1 mM EDTA, pH 7.2. The filter was washed in 2x SSC (20 x SSC = 0.3M tri-sodium citrate, 3M NaCl, pH 7.0) and dried before baking (80 °C; 2 h). The filter with the DNA fixed onto its surface was washed twice in 1M NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 50 mM Tris-HCl, pH 8.0 and then incubated (1 h; 37 °C) with 10 ml proteinase K solution (2x SSC, 0.1% (w/v) SDS, 50 $\mu g\ ml^{-1}$ proteinase K) with constant shaking. The proteinase K-treated filter was washed three times in 5x SSC (5 min; 37 °C).

B.1.3 Non-alkaline lysis for detecting RNA target sites

The 16S rRNA probes hybridise to RNA target sites within the bacterial cells. RNA is rapidly hydrolysed in alkaline solutions and lysis of cells using NaOH was not therefore appropriate. The strong anionic detergent sodium dodecyl sulphate (SDS) was used as a cell membrane lysing agent as described in the method of Ivanov and Gigova (1986) for preparing RNA in colonies for hybridisation to gene probes. SDS would also disrupt and denature the ribosomes in which the 16S rRNA molecules are sequestered. Filters were placed for 10 min on filter pads soaked in 10% (w/v) SDS to lyse the bacteria in the colonies. Ivanov and Gigova (1986) recommended washes on pads soaked in 3 x SSC at 65 °C. The pH of 10% (w/v) SDS solution was slightly acidic which tends to stabilise RNA. The pH, however, of 3x SSC was slightly alkaline which promotes RNA degradation. Washing the filters in 3 x SSC was therefore omitted, and after lysis on SDS-soaked pads, the filters were transferred to an oven (2 h; 75 °C) to fix the nucleic acid to the surface.

B.2 Hybridisation of gene probes to membrane filters and control of stringency

The process by which the gene probe binds to DNA or RNA target sites on the nitrocellulose filters is called hybridisation. The conditions for hybridisation varied depending on whether the DNA probe was an oligonucleotide (e.g. Ec1000) or a gene probe (e.g. *lac* gene probe). Thus, hybridisation was performed for 1 h with HRP-labelled Ec1000 while longer gene probes, such as HRP-labelled *lac* gene probe, required an overnight hybridisation. Furthermore, hybridisation protocols varied depending on the nature of the non-radioactive label. Thus, the temperatures for hybridisation and washing steps using HRP-labelled probes must not exceed 42 °C since the HRP will be denatured and inactivated. Hybridisation protocols were performed as stipulated in the instructions provided with the different non-radioactive labelling kits.

After hybridisation of the probe to target DNA, the filters were washed. The washing steps are used to increase the stringency of hybridisation so that cross-reaction of a probe with DNA from closely related species may be eliminated. Stringency of hybridisation may be increased by varying three parameters:

- Increasing the temperature reduces the ability of mismatched probes to bind to potential DNA target sites.
- Decreasing the concentration of SSC in the hybridisation buffer and washing buffers.
- Adding urea up to a concentration of 6M reduces binding of mismatched probes to DNA target sites.

Hybridisation and washing conditions for the ECL 5'-oligolabelling, ECL 3'-oligolabeling, ECL gene probe labelling and DIG-DNA kits are described below.

B.2.1 Hybridisation and washing protocols for the DIG-DNA gene probe labelling and detection kit

A volume of 4 ml of DIG hybridisation buffer (5x SSC, 1% (w/v) blocking reagent (supplied by Boehringer), 0.1% (w/v) N-lauroylsarcosine sodium salt, 0.02% (w/v) SDS) was added to the filter and shaken (1 h; 68 °C). The DIG-labelled probe was heated (95 °C; 10 min), cooled and added to 1 ml of hybridisation buffer. Hybridisation was performed (16 h; 68 °C) in a shaking water bath. The filter was washed twice in 15 ml DIG primary wash buffer (2 x SSC, 0.1% (w/v) SDS) by shaking in a water bath (5 min; 20 °C). The filter was then washed twice in 15 ml of DIG secondary buffer (0.1x SSC, 0.1% (w/v) SDS) for 15 min at 68 °C. The filter was shaken in 20 ml of DIG buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 min at 20 °C. The filter was shaken in 20 ml of DIG buffer 2 (buffer 1 with 1% (w/v) DIG blocking agent) for 30 min at 20 °C. The filter was incubated in 4 ml of antibody-alkaline phosphatase conjugate (30 min; 20 °C). The filter was washed twice in 20 ml DIG buffer 1 (15 min; 20 °C) and then 4 ml DIG buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) (2 min; room temperature). The filter was soaked in 2 ml of 'DIG colour solution' (45 µl of 70% (v/v) nitroblue tetrazolium salt solution, 35 µl of 50 mg ml^{-1} 5-bromo-4-chloro-3-indoyl phosphate, toluidinium salt solution, dissolved in 10 ml buffer 3). Colour development was allowed to proceed in the dark (90 min). The reaction was stopped by shaking the filter with 10 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 8) for 5 min. The filter was wrapped in clingfilm and photocopied.

B.2.2 Hybridisation and washing protocols for the ECL 3'-oligonucleotide probe labelling and detection kit

Volumes in this protocol apply to filters which are 47 mm in diameter. The filter was shaken in 50 ml 2x SSC. The filter was removed and soaked in 5 ml hybridisation buffer (5x SSC, 0.1% (w/v) hybridisation buffer component (supplied by Amersham), 0.02% (w/v) SDS, 0.5% (w/v) blocking agent (supplied by Amersham)) for 30 min at 42 °C. Probe (Ec1000) labelled at the 3'-end with a fluorescein-nucleotide was added to the hybridisation buffer and hybridisation performed for 1 h at 42 °C in a shaking water bath. The filter was washed twice in primary wash buffer (5x SSC, 0.1% (w/v) SDS) for 5 min at room temperature and then twice in secondary wash buffer (1x SSC, 0.1% (w/v) SDS) at 42 °C. The SSC concentration of the secondary wash buffer may be reduced to 0.1x SSC to enhance the stringency of hybridisation. The filter was incubated in 40 ml buffer 1 (0.15 M NaCl, 0.1M Tris-HCl, pH 7.5) for 1 min at room temperature. The filter was then soaked in 5 ml block solution (buffer 1 with 0.5% (w/v) blocking agent) for 30 min at room temperature. The filter was washed in 40 ml of buffer 1 (1 min; room temperature) and then incubated with 5 ml antibody-HRP conjugate (30 min; room temperature). The filter was washed three times in 40 ml of 0.4M NaCl, 0.1M Tris-HCl, pH 7.5) for 5 min at room temperature. Light detection on photographic film was then performed.

B.2.3 Hybridisation and washing protocols for the ECL 5'-oligonucleotide probe labelling and detection kit

Volumes in this protocol apply to filters which are 47 mm in diameter. Each filter was prehybridised in 5 ml of hybridisation buffer (5x SSC, 0.5% (w/v) blocking reagent (supplied by Amersham), 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS) for at least 15 min at 42 °C. 5'-end HRP-labelled oligonucleotide probe (Ec1000) was added to a concentration of 20 ng ml^{-1} of hybridisation buffer. Filter hybridisations were typically carried out at 42 °C for 1 h with gentle shaking. The filter was washed twice in 40 ml 3x SSC, 0.1% (w/v) SDS (42 °C, 15 min). Stringency was controlled during the washing steps by the addition of formamide or urea, or by increasing the temperature (up to a maximum of 65 °C) in the second of the two 3x SSC, 0.1% (w/v) SDS washes. The filter was soaked in excess 2x SSC (5 min; room temperature). Light detection on photographic film was then performed.

B.2.4 Hybridisation and washing protocols for the ECL gene probe labelling and detection kit

Amersham supply ready made hybridisation buffer with the kit. NaCl is added to a concentration most suitable for effective hybridisation of the probe. The optimum hybridisation conditions may be different for different probes and for the *lac* gene probe a concentration 0.5M was used. Blocking agent was added to a concentration of 5% (w/v). The filter was placed in the buffer and prehybridised for at least 15 min at 42 °C. HRP-labelled *lac* gene probe was added to the hybridisation buffer and hybridisation continued overnight at 42 °C. Filters were washed twice in primary wash buffer (6M urea, 0.5x SSC, 0.4% (w/v) SDS) for 20 min at 42 °C. Stringency may be altered by decreasing the SSC concentration in the primary wash buffer down to 0.1x SSC. Filters were washed in an excess of 20x SSC. Light detection on photographic film was then performed.

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APPENDIX C DETECTION OF TOTAL COLIFORMS IN WATER SAMPLES USING MEMBRANE FILTRATION AND THE *LAC* GENE PROBE

C.1 Development of a total coliform specific gene probe

Total coliforms are defined as lactose-positive members of the *Enterobacteriaceae*. Lactose utilisation by a coliform bacterium requires two proteins (β-galactosidase and lactose permease) which are coded for by a group of genes collectively called the *lac* operon. University of Leicester thus selected the *lac* operon as a target site for a potential coliform specific probe.

C.2 Species-specificity of the *lac* operon gene probe

University of Leicester purified a 2.18 kbp restriction fragment from the commercial plasmid, pPM18. This fragment contained part of the β-galactosidase gene and after labelling with radioactive ^{32}P was used as a probe for total coliform organisms. Species specificities for this probe for a number of coliform and non-coliform *Enterobacteriaceae* are presented in Table C.1, together with the ability to produce acid and gas from membrane lauryl sulphate broth at 37 °C. With the exception of three species which produced acid only, there was agreement between *lac* probe binding and production of acid and gas from membrane lauryl sulphate.

C.3 Detection of total coliform colonies with the *lac* gene probe

Initial studies to assess the ability of the *lac* gene probe to detect coliform colonies were performed using the ^{32}P-labelled probe by University of Leicester. WRc assessed the use of the *lac* gene probe labelled either with the Boehringer DIG kit or with the Amersham enhanced chemiluminescent (ECL) kit. These results are now presented.

C.3.1 ^{32}P-labelled *lac* gene probe

Initial protocols implemented by University of Leicester to enumerate total coliform colonies using membrane filtration and the *lac* gene probe labelled with ^{32}P required approximately 40 h. A water sample spiked with *E. coli* was filtered. The filter was cultured for 8 h on nutrient broth. Filters were treated with NaOH to lyse the colonies and baked to fix the DNA to the nitrocellulose. Hybridisation was performed (5 h) and radio-labelled colonies were strongly detected by autoradiography after 18 h. The protocol is summarised in Table C.2.

Table C.1 Species specificity of *lac* probe (labelled with ^{32}P) compared with production of acid and gas from membrane lauryl sulphate broth (37 °C) for a number of coliform and non-coliform *Enterobacteriaceae*. Results were reported by University of Leicester

Organism	Hybridisation of *lac* probe	Acid and gas from MLSB
Buttiauxella agrestis	+	+
Cedecea davisea	+	A
Citrobacter freundii	+	+
Edwardsiella tarda	-	-
Enterobacter aerogenes	+	+
Enterobacter cloacae	+	+
Escherichia coli	+	+
Ewingella americana	+	A
Hafnia alvei	+	+
Klebsiella aerogenes	+	+
Klebsiella edwardsii	+	+
Klebsiella pneumoniae	+	+
Koserella trabulsii	+	A
Leminorella grimontii	-	-
Mollerella wisconsensis	-	A
Proteus morganii	-	-
Proteus vulgaris	-	-
Providencia alcalifaciens	-	-
Serratia marcescens	-	A
Tatumella ptyseos	-	-

A = acid positive, no gas

C.3.2 *Lac* gene probes labelled with non-radioactive markers

BluGENE biotin label

University of Leicester found that only 2 h were required for the detection of biotin-labelled probes, compared to the 18 h for autoradiography of ^{32}P-labelled probes (Table C.2). Using the non-radioactive biotin detection procedure, coliform colonies could be enumerated and confirmed with the *lac* gene probe in 16 h. However, the rapid detection probably reflected non-specific detection due to endogenous biotin within the cells (Appendix D.3).

Table C.2 Procedure used by University of Leicester for detection of total coliform colonies using the *lac* gene probe. Times taken for each step are indicated

Step	Time taken (h)
Filter water sample	
Incubate	8
Lyse colonies	0.25
Air dry and bake	2.50
Prehybridise	2.00
Hybridise	5
Wash	3
Autoradiography	18

HRP-labelled *lac* gene probe

University of Leicester provided a 2.18 kbp *lac* probe purified from the commercially available plasmid, pPM18. A eucaryotic DNA plasmid (pCH110) was purchased from Pharmacia. This plasmid contained a functional *lac* operon and would also serve as a gene probe for total coliforms. These DNA sequences were labelled with HRP using the Amersham gene probe ECL kit to produce HRP-labelled *lac* gene probes which should detect total coliforms.

HRP-labelled pCH110 bound strongly to commercially available *E. coli* genomic DNA extract which had been 'dotted' and immobilised onto a nitrocellulose filter (DNA dot blot). As little as 125 ng of *E. coli* genomic DNA was detectable. Commercial DNA extracts of genomic DNA from *Clostridium perfringens* were not detected. These observations confirmed that the HRP-labelled *lac* gene probe not only bound DNA with correct specificity but also could be detected.

Cultures isolated from the environment were grown up into colonies on membrane filters. Colonies were lysed with NaOH. In initial experiments, lysed colonies were not treated with proteinase K prior to hybridisation with the HRP-labelled probes. The HRP-labelled *lac* gene probe was found to bind to colonies of *Vibrio fluvialis* as strongly as to those of *E. coli*. While *E. coli* is a coliform, *Vibrio fluvialis* cannot ferment lactose and is a non-coliform. Thus, the *lac* gene probe should not detect *Vibrio fluvialis* colonies. HRP-labelled pCH110 also bound to colonies of two other 'non-lactose fermenting' bacteria. These results suggested that the HRP-label on the *lac* gene probe may be responsible for non-specific binding to proteinaceous material in the debris remains of the lysed colony, since the ^{32}P-labelled *lac* gene probe selectively bound to coliforms and not to non-coliforms. Non-specific binding by HRP was confirmed in experiments in which the HRP provided in the ECL gene probe kit was used alone i.e. not linked to a *lac* gene

probe. The HRP enzyme alone bound strongly to colonies of both *E. coli* and *Vibrio fluvialis*.

Treatment of the filters with proteinase K after colony lysis using the method described by Riley and Caffrey (1990) (Appendix B.1.2) removed much of the colony debris and appeared to overcome the non-specific binding by the HRP. Thus, colonies of *E. coli* showed strong signals on labelling by the HRP-labelled pCH110 *lac* probe after proteinase K treatment. In contrast, colonies of *Aeromonas hydrophila* were not labelled. The total time taken for enumeration of coliforms from water samples with the HRP-labelled *lac* probe was 42 h. The major time-consuming steps were an 18 h incubation to culture up colonies on the membrane filter and an 18 h hybridisation with the gene probe.

DIG-labelled *lac* gene probe

The DNA from the *lac* operon plasmid pCH110 was labelled with digoxigenin using the DIG-DNA kits. Binding specificity and detection of the DIG-labelled *lac* gene probe was tested by DNA dot blots. pCH110 DNA immobilised on nitrocellulose filters was strongly detected, which demonstrated that the probe was functional. The probe also bound strongly to M13mp8 DNA, which was expected since it contains part of the *lac* operon. No signal was detected from phage lambda DNA which served as a negative control, although in some experiments a slight cross-reaction was observed. Commercially available *E. coli* genomic DNA was also detected. It was found that 20 pg of pCH110 DNA and 3.58 ng of *E. coli* DNA were readily detected by the DIG-labelled *lac* gene probe.

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APPENDIX D DETECTION OF *E. COLI* IN WATER SAMPLES USING MEMBRANE FILTRATION AND THE 16S rRNA PROBES

D.1 Development of *E. coli* specific gene probes for 16S rRNA

Development of a gene probe for *E. coli* was more difficult than for total coliforms (Appendix C.1) because proteins unique to *E. coli* are less well defined than those of the *lac* operon of total coliforms. One enzyme on which several defined substrate technology methods for *E. coli* are based is β-glucuronidase (Edberg *et al.* 1988) which hydrolyses 4-methylumbelliferyl-β-D-glucuronide (MUG) and is responsible for the MUG phenotype. Bej *et al.* (1991) have developed a PCR method using primers specific for the β-glucuronidase gene. However, some *E. coli* strains have a MUG negative phenotype (Chang *et al.* (1989)) although Bej *et al.* (1991) report that their PCR method for *E. coli* detection appears to detect MUG negative strains that may constitute 15% of the *E. coli* in the waters tested and that are not detected by the defined substrate technology methods.

The approach adopted by University of Leicester was to identify unique sequences to *E. coli* within the RNA molecules which comprise cellular structures called ribosomes. One of these RNA molecules called the 16S ribosomal RNA (rRNA) was selected because its nucleotide sequence (Ehresmann *et al.* 1977) differs from species to species. 16S rRNA molecules contain regions of conserved sequence which are the same in different bacterial species and also regions of variable sequence which are specific to a particular species. The strategy used by University of Leicester was to compare nucleotide sequences for 16S rRNA molecules from *E. coli* with those from a variety of related bacteria. These sequences were not available in the literature and much of the initial work performed by University of Leicester concentrated on sequencing 16S rRNA molecules from bacteria including *Enterobacter aerogenes*, *Enterobacter cloacae*, *Hafnia alvei*, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *Buttiauxella agrestis*, *Citrobacter freundii*, *Proteus morganii*, *Cedecea davisae* and *Koserella trabulsii*. The 16S rRNA from *E. coli* is over 1500 nucleotides long and University of Leicester sequenced selected regions of 150-200 nucleotides using four primers which started at bases 536, 690, 1100 and 1390, respectively. Thus, partial sequences of variable regions for 16S rRNAs were obtained for a number of coliform and non-coliform organisms. Comparison of sequences between species showed the majority of sequences were similar. Nucleotide differences, however, were identified within the variable regions and it was these that offered potential for development of *E. coli* specific probes.

University of Leicester supplied WRc with four oligonucleotide sequences, which on the basis of nucleotide sequence differences would serve as *E. coli* specific probes. These are presented in Table D.1.

Table D.1 Nucleotide sequences for four *E. coli* specific 16S rRNA oligonucleotide probes supplied by University of Leicester. Ec1000 is incorrect in that two nucleotide bases located five bases from the 3'-end were omitted by University of Leicester when they reported the sequence

Name	Sequence	Number of bases
Ec1000	5'-ATTCTCATCTCTGAAAACTTCCG-3'	23
Ec636	5'-AGCTTGCCAGTATCAG-3'	16
Ec186	5'-AAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGC-3'	33
Ec825	5'-GCCTCAAGGGCACAACCTCCAAG-3'	23

D.2 Effect of incorrect Ec1000 nucleotide sequence on detection of *E. coli* colonies

Ec1000 was suggested by University of Leicester to be the most promising *E. coli* specific probe and they evaluated species specificity and potential for detecting *E. coli* colonies with this probe. WRc also performed much evaluation of the ability of Ec1000 to detect colonies after culture on membrane filters. Unfortunately, the sequence for Ec1000 which was taken from that published by Ehresmann *et al.* (1977) was misread by University of Leicester. Indeed the 16S rRNA sequence for *E. coli* (from Ehresmann *et al.* 1977) which University of Leicester entered into their computer for comparison with the 16S rRNA sequences from other bacteria contained a deletion of two nucleotides. This deletion occurred five nucleotides from 3'-end of the Ec1000 sequence provided by University of Leicester. The consequences of this are twofold:

- The sequence used by WRc would have a lower binding potential for *E. coli* DNA than expected. This would perhaps explain the low sensitivity for binding of Ec1000 to *E. coli* colonies.

- University of Leicester aligned the sequences they obtained for other bacteria with the published sequence for *E. coli* 16S rRNA, which they entered incorrectly. They selected Ec1000 on the basis of sequence variation at this region. Incorrect entry of the sequence may have contributed to this sequence variation. Thus, Ec1000 may not be an *E. coli* specific probe.

The nucleotide sequence for the correct Ec1000 is presented below:

5'-ATTCTCATCTCTGAAAACACTTCCG-3'

This error was not discovered by WRc until the final stages of the contract.

Thus, virtually all the work on detection of *E. coli* colonies was performed with a gene probe with a two base pair deletion. This could reduce to some extent the efficiency of probe binding to *E. coli* genomic material, particularly under high stringency hybridisation conditions. However, it is unlikely that the error will eliminate binding altogether. Indeed, there was clearly binding of Ec1000 to *E. coli* DNA and results which will prove useful in implementing gene probe technology in the water industry have been obtained are reported.

Binding of the correct Ec1000 probe sequence to its target site is shown below and compared with binding of the Ec1000 probe sequence supplied by University of Leicester.

Correct sequence

```
      5'-ATTCTCATCTCTGAAAACACTTCCG-3'              probe
3'----------UAAGAGUAGAGACUUUUGUGAAGGC----------5'  16 rRNA
```

Sequence supplied by University of Leicester

```
      5'-ATTCTCATCTCTGAAAAC**TTCCG-3'              probe
3'----------UAAGAGUAGAGACUUUUGUGAAGGC----------5'  16 rRNA
```

** represent the two missing bases.

It is likely that the error will have little impact on binding of Ec1000 labelled at the 3'-end because the 18 nucleotides at the 5'-end of the probe match the target sequence. Indeed the Amersham 3'-oligolabelling kit adds fluorescein-labelled nucleotides to the 3'-end. However, the error would be expected to reduce binding of probes labelled at the 5'-end with HRP.

D.3 Species-specificity of the 16S rRNA oligonucleotide probes

Since their probe sequence was incorrect, results of evaluations of species-specificities of Ec1000 by University of Leicester were of no value. However, by serendipity their experiments demonstrated the inadequacy of the biotin-labelling kits for colony blots. University of Leicester investigated the specificity of binding of Ec1000 to colonies of a number of *Enterobacteriaceae* using probes which were labelled with biotin. University of Leicester experienced a problem with the biotin-labelled Ec1000 in that not only were *E. coli* colonies detected but also colonies from a variety of other species. Indeed five species of non-coliform *Enterobacteriaceae* were also detected. University of Leicester did not realise the Blu-GENE biotin detection system was giving false positives from endogenous biotin present in the cells. Most cells contain biotin as a requirement for certain enzymes. The biotin detection kit was designed for purified DNA systems and should not be applied to the detection of bacterial colonies.

At this stage the contract with University of Leicester ended and the project was continued at WRc Medmenham. Species specificity studies on Ec1000 were performed

on commercial DNA extracts (Sigma) using the Amersham 3'-oligonucleotide labelling kit. The commercial DNA extracts contain purified DNA and are free from bacterial proteins and other debris which might have contributed to non-specific binding. At low stringency hybridisation conditions (1x SSC, 0.1% (w/v) SDS, 42 °C), Ec1000 at a probe concentration of 10 ng ml^{-1} in the hybridisation buffer bound very strongly to *E. coli* DNA but also showed a weak cross-reaction with DNA from *Clostridium perfringens*. DNA from *Micrococcus lysodeikticus* and from the virus M13 showed no cross reaction with Ec1000. Under such low stringency hybridisation conditions (1x SSC, 0.1% (w/v) SDS, 42 °C) one of the poliovirus PCR primers, PG2 (Appendix E.2) was observed to bind strongly to DNA from all three bacterial species. This was unexpected and suggested a chance similarity between a sequence in poliovirus genomic RNA and sequences in DNA from *E. coli*, *Clostridium perfringens* and *Micrococcus lysodeikticus*. Increasing the stringency of hybridisation by urea washes (1x SSC, 0.1% (w/v) SDS, 6M urea, 42 °C) eliminated binding of the probe PG2 to DNA from all three bacteria. Binding of Ec1000 to *E. coli* DNA, however, was little affected by the presence of urea in the washes. There is some evidence that urea reduced the cross-reaction of Ec1000 with *Clostridium* DNA, although the effect was less clear than for PG2 binding. The effects of varying the temperature and SSC concentration on Ec1000 binding to *E. coli* DNA were also investigated. At a temperature of 55 °C in 0.1x SSC binding of Ec1000 to *E. coli* DNA was virtually eliminated. At 55 °C in 1x SSC, however, *E. coli* DNA was strongly labelled by Ec1000 and the cross-reaction with *Clostridium* DNA was eliminated. These experiments suggested that by varying the urea and SSC concentrations and the wash temperatures, optimal conditions for Ec1000 binding could be identified such that *E. coli* DNA was labelled to the exclusion of DNAs from other species.

D.4 Elimination of non-specific detection of colonies by the Amersham ECL oligonucleotide detection kits

Initial results of colony detection with HRP-labelled Ec1000 appeared encouraging. Presumptive *E. coli* colonies cultured up for a period of 18 h (4 h, 30 °C; 14 h, 44 °C) and labelled with HRP-Ec1000 were detectable after a 3 min exposure to photographic film. It was found that "micro-colonies" could be detected with HRP-labelled Ec1000 after as little as 6 h culture on membrane lauryl sulphate broth (35 °C). To compensate for the smaller colony sizes a longer exposure (4 h) to the photographic film was necessary. The total detection time was 11 h 10 min. However, binding of HRP-labelled Ec1000 to these colonies was due to non-specific binding of the the enzyme HRP to proteinaceous debris on the colony remains. Evidence for this was two-fold.

- HRP alone bound strongly to lysed colonies.
- HRP-labelled Ec1000 detected colonies from a variety of environmental isolates other than *E. coli*. These included *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Vibrio fluvialis* and *Aeromonas hydrophila*. This was not due to DNA-DNA hybridisations since including 6M urea in the washing steps did not eliminate non-specific binding.

Although Amersham recommended the use of their ECL 5'-oligonucleotide labelling and detection system for screening colonies they were aware of the problem of non-specific binding to colony debris by HRP. Amersham suggested several possible treatments to overcome specific binding of HRP in the labelled Ec1000 probes. These included treatment of the HRP-labelled probe sample with a thiol reagent prior to hybridisation, and removal of colony debris prior to hybridisation. For some HRP-labelled probe samples, treatment with thiol reagents including mercaptoethanol, dithiothreitol, or mercaptoethylamine, prior to hybridisation reduced but did not completely eliminate non-specific labelling of colonies. HRP alone after treatment with mercaptoethylamine still bound to colony debris. Amersham admitted that HRP is a 'sticky' protein and that removal of colony debris from the surface of the filter prior to hybridisation was necessary. Amersham suggested two methods to remove colony debris. Physical rubbing of the filter surface after the prehybridisation step prior to hybridisation with the HRP-labelled gene probe showed little improvement. However, treatment of the filter with proteinase K (Riley and Caffrey 1990) after baking to fix the DNA to the surface was successful. Thus colonies of *E. coli* were labelled selectively with HRP-labelled Ec1000 in contrast to those from *Vibrio fluvialis*. Similarly the probe Ec825 labelled with HRP appeared to label colonies of *E. coli* in preference to those of *Enterobacter cloacae*.

D.5 Detection of *E. coli* colonies on membrane filters by Ec1000 and the 3'-oligonucleotide labelling and detection system

Using Ec1000 labelled with the 3'-oligolabelling and detection kit it was possible to detect *E. coli* colonies grown up on membrane filters from inoculations of bacteria isolated from environmental samples. To prevent non-specific binding of the HRP protein the colonies were treated with proteinase K (Riley and Caffrey 1990) after alkaline lysis. Essentially the method to detect *E. coli* colonies with the 16S rRNA probe, Ec1000, appears to be working. *E. coli* colonies generated stronger signals when hybridised with the probe than those of *Aeromonas hydrophila*, while *Citrobacter freundii* colonies were not detected. However, signal intensity from the colonies was weak and *E. coli* colonies grown from filtered water samples were not detectable. Only the larger colonies obtained from the neat cultures were detectable with Ec1000. Furthermore only low stringency wash conditions could be implemented. Thus, in 3x SSC strong signals from *E. coli* colonies were obtained. With 2x SSC in the final wash weaker signals were obtained, while with 1x SSC (recommended by Amersham) no signal was observed.

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APPENDIX E USE OF THE POLYMERASE CHAIN REACTION TO DETECT VIRUSES IN WATER SAMPLES

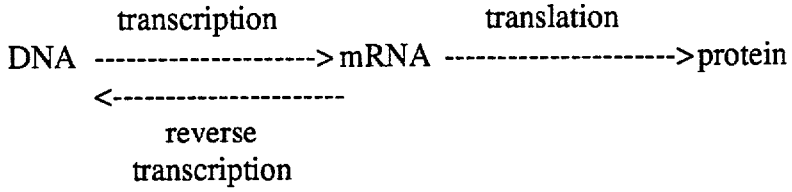
E.1 Molecular Biology of Waterborne Viruses

The enteroviruses of major health significance to the water industry contain RNA genomes. The molecular biology of two groups of enteroviruses which are responsible for several waterborne illnesses is described. These are the picornaviruses and the rotaviruses.

E.1.1 Picornaviruses

Poliovirus is a member of the group of viruses called picornaviruses, which also includes rhinoviruses (common cold) and hepatitis A virus (responsible for infectious hepatitis). Both poliovirus and hepatitis A virus are transmitted in water through the faecal-oral route. Picornaviruses are characterised by small size (PICOrna) and the presence of a single-stranded RNA molecule (picoRNA) for the genome. The RNA is surrounded by a coat comprised of four different subunits. The 5' end of the viral RNA, which for poliovirus is 7500 nucleotides long, is covalently linked to a peptide (VPg). Complete nucleotide sequences are available for poliovirus serotypes 1, 2 and 3. The nulceotide sequence is also available for hepatitis A virus.

The RNA genome of picornaviruses is said to be positive (+) sense, which means that direct translation at the ribosomes produces the proteins coded for by the genetic material. Thus, poliovirus genome RNA resembles messenger (m)RNA which is the nucleic acid intermediate between DNA and protein. The mRNA is translated in the cell cytoplasm at the ribosomes to produce cellular proteins.



In common with cellular mRNAs, the poliovirus RNA molecule contains a tract of approximately 200 adenine (A) bases at its 3'-end. This is called a poly A tail.

E.1.2 Rotaviruses

Rotavirus is the most common in the group of viruses which cause gastroenteritis. The genome of rotaviruses is comprised of 11 segments of double stranded RNA. On infecting cells, the double-stranded RNA is transcribed into messenger RNAs (mRNA) which code for the proteins required for virus production.

E.2 Development of gene probes and PCR primers for waterborne viruses

Sequences of the upstream (PG2) and downstream (PG1) primers for PCR amplification of poliovirus RNA were obtained from University of Warwick and are presented in Table E.1. At one site on each probe there is variation between the three poliovirus serotypes and to ensure that the primers amplified RNA from all serotypes half of the probes were synthesised with a G instead of an A at the sites shown in Table E.1. A 20-base sequence on the (-) polarity DNA strand of the 530 base pair amplified PCR product was chosen as a target site and the complementary oligonucleotide labelled at the 5'-end with biotin was purchased from British Biotechnology for use as a probe. The sequence for this probe is presented in Table E.1.

Table E.1 Nucleotide sequences of PCR primers designed for amplification of a 530 base pair DNA fragment from the poliovirus RNA genome. The sequence of a biotin-labelled probe for detection of the amplified DNA on gels is also presented

PG1	5'-GCCTT G/A CCACACACTAAAGG-3'	downstream primer
PG2	5'-CAGTTCAAGAGCAA G/A CACCG-3'	upstream primer
Probe	5' biotin--CTACCCCCGGATCCATCACA 3'	

Sequences of upstream (G1) and downstream (G2) primers for PCR amplification of bovine rotavirus RNA were also obtained from University of Warwick and are shown in Table E.2.

Table E.2 Nucleotide sequences for PCR primers for amplification of bovine rotavirus RNA

G1	5'-CCGTTTGGCTAGCGGTTAGCT-3'	upstream primer
G2	5'-TTGACCTTTTTTACCACCGTTCACAA-3'	downstream primer

Poliovirus and rotavirus genomic material is RNA; single-stranded RNA of positive polarity for poliovirus and double-stranded RNA for rotavirus. Before RNA viruses may be detected by PCR their genetic material must be converted to DNA by the process of reverse transcription. The genetic material of adenovirus is DNA and not RNA. Adenovirus may therefore be detected by direct PCR of the DNA, i.e. without reverse transcription. Adenovirus DNA with two appropriate PCR primers was used as a control for amplification of the DNA by PCR. Adenovirus-2 DNA was purchased from Sigma (D 3390). The sequences of the upstream (A1) and downstream (A2) primers for PCR amplification were obtained from University of Warwick and are shown in Table E.3. The primers amplify a 411 basepair DNA sequence.

Table E.3 Nucleotide sequences for PCR primers for amplification of a 411 basepair sequence from adenovirus-2 DNA

A1	5'-CGAGCCAATATGATAATGAGTG-3'	upstream
A2	5'-GAACTCACGGTCGCTCATCTC-3'	downstream

E.3 Methods for detection of viruses in water samples using the polymerase chain reaction

E.3.1 Methods for extraction and concentration viral genomic material from virus cultures

Virus culture

An attenuated vaccine strain from poliovirus type 2 was cultured in a Buffalo Green Monkey cell line until a cytopathic effect was observed and viruses were harvested by centrifugation and stored at -80 °C.

Confluent monolayers of LLCMK2-cells (rhesus monkey kidney) were used for the production of rotavirus. Monolayers were grown in glass/plastic roller bottles, in a growth medium containing foetal calf serum, at 37 °C. Growth medium was removed, the cells washed once with serum-free medium and culture flasks/bottles incubated with serum-free medium at 37 °C for 1 h. Growth medium was removed and bovine rotavirus suspension added. Flasks were incubated at 37 °C for 1 h. Serum-free growth medium was added and flasks incubated until a cytopathic effect was visible (i.e. when 75% were showing a cytopathis effect). The viruses were harvested by shaking the medium and cells and freezing down to -80 °C. Cells were frozen and thawed three times and the cells precipitated by centrifuging at low speed. The supernatant containing the viruses was stored at -80 °C.

Extraction of viral RNA

RNA was extracted from poliovirus and rotavirus cultures using 8M guanidine HCl. A volume of 5.0 ml of guanidine HCl homogenisation buffer I (8 M guanidine HCl, 0.1 M sodium acetate, 0.5 mM dithiothreitol, 0.5% (w/v) sodium lauryl sarcosinate, pH 5.2) was added to 0.5 ml of virus suspension and shaken for 5 min at room temperature. This was then centrifuged (5000 g; 10 min; room temperature) to remove debris. A volume of 0.5 ml of 3M sodium acetate (pH 5.2) was added to the supernatant and nucleic acid precipitated by the addition of 3 ml ice cold ethanol. Samples were incubated at -20 °C for at least 2 h. Nucleic acid was recovered by centrifuging (5,000 g; 0 °C; 10 min). The pellet was dissolved in 5 ml guanidine HCl homogenisation buffer II (8 M guanidine HCl, 0.1 M sodium acetate (pH 7.0), 1 mM dithiothreitol, 20 mM EDTA (pH 8.0)) and 2.5 ml of ice cold ethanol was added. Samples were incubated at -20 °C for at least 2 h and centrifuged (5000 g; 10 min; 0 °C). The pellet was resuspended in homogenisation buffer II. Ethanol precipitation and resuspension in ethanol was repeated twice and the precipitated nucleic acids frozen until used for PCR. The guanidine not only disrupts the virion proteins so releasing the encapsidated RNA but also denatures any ribonuclease enzymes, thus protecting the naked RNA against enzymatic degradation. Ribonucleases are stable enzymes and are present on skin and probably in cell cultures.

It has been reported (Richardson *et al.* 1988) that heat-treatment (65 °C; 30 min) releases or exposes poliovirus RNA genomes. The elevated temperature (70 °C) would denature any RNAase enzymes present. Heat-treatment immediately prior to PCR amplification was used to disrupt the poliovirus particles, avoiding the lengthy RNA extraction procedures described above.

E.3.2 Amplification of viral RNA by the polymerase chain reaction (PCR)

RNA PCR kits were obtained from Perkin Elmer Cetus. Perkin Elmer Cetus market two different RNA PCR kits; the rTth kit and the retrovirus reverse transcriptase (RTase) kit. Both were used in attempts to amplify poliovirus and rotavirus RNA sequences.

The rTth RNA PCR kit

Initially the rTth kit was used, although this kit is harder to operate (Perkin Elmer Cetus, personal communication) than the RTase kit. The rTth kit was chosen because it operates at a much higher temperature than the RTase kit. Higher temperatures were required to eliminate possible secondary structures within the poliovirus and rotavirus genomic RNAs. The rTth kit uses the thermostable rTth polymerase enzyme. In the presence of Mn^{++} ions the rTth polymerase performs reverse transcription of the viral RNA using the downstream primer to produce cDNA. The Mn^{++} ions were removed by chelation and replaced by Mg^{++} ions. In the presence of Mg^{++} ions the rTth polymerase has DNA dependent DNA polymerase activity and the cDNA is amplified by conventional PCR using both upstream and downstream primers.

Three mixes were made up as multiples of the following volumes:

Mix A	sterile distilled water	6.4 µl
	10 x rTth RTase buffer	2.0 µl
	Downstream primer (0.015 nmole)	1.0 µl
	virus RNA	5.0 µl
Mix B	10 mM $MnCl_2$ soln	2.0 µl
	dGTP	0.4 µl
	dATP	0.4 µl
	dTTP	0.4 µl
	dCTP	0.4 µl
	rTth polymerase	2.0 µl
Mix C	sterile distilled water	65.0 µl
	10 x chelating buffer	8.0 µl
	$MgCl_2$ soln (25 mM)	6.0 µl
	Upstream primer (0.015 nmole)	1.0 µl

A volume of 14.4 µl of Mix A was added with 5.6 µl of Mix B to a 0.5 ml PCR tube and spun in a microfuge. Mineral oil was added and the tube maintained at 70°C for 10 min (using a soak file on the Perkin Elmer Cetus PCR thermal cycler), allowing reverse transcription of the viral RNA with the downstream primer to proceed. The tubes were placed on ice and 80 µl of Mix C added. The chelating agent in Mix C removes the Mn^{++} ions and in the presence of 1.5 mM Mg^{++} ions the rTth polymerase adopts a DNA polymerase activity allowing PCR amplification of the cDNA using both upstream and downstream primers. The PCR amplification was performed in the thermal cycler using a step-cycle with following parameters:

Segment 1	Denaturation	Temp 95 °C Time 1 min
Segment 2	Primer annealing	Temp 37 °C Time 1 min
Segment 3	Primer extension	Temp 60 °C Time 1 min + 30 sec extension per cycle

After 35 cycles the temperature of the block was set to 4 °C.

The RTase RNA PCR kit

The RTase RNA PCR kit is reported to be easier to use than the rTth kit. It contains two enzymes. Retrovirus reverse transcriptase converts the viral RNA to cDNA using the downstream PCR primer. A thermostable DNA polymerase, purified from the thermophilic bacterium, *Thermus aquaticus*, then performs amplification of the cDNA

products by conventional PCR. Two reaction mixes were made up as multiples of the following volumes:

Mix A	$MgCl_2$	4.0 µl
	10 x PCR buffer	2.0 µl
	Sterile distilled water	1.0 µl
	dGTP	2.0 µl
	dATP	2.0 µl
	dTTP	2.0 µl
	dCTP	2.0 µl
	RNase inhibitor	1.0 µl
	Reverse transcriptase	1.0 µl
Mix B	$MgCl_2$	4.0 µl
	10 x PCR buffer	8.0 µl
	Sterile distilled water	65.5 µl
	AmpliTaq DNA polymerase	0.5 µl

Downstream primer (1.0 µl, to give a final concentration of 0.15 µM) and target RNA (2.0 µl) were added to 17.0 µl of Mix A in a PCR tube. Mineral oil was added to each tube to prevent evaporation. Reverse transcription was carried out on the Perkin Elmer Cetus thermal cycler. Samples were incubated at 42 °C for 15 min and then 99 °C for 5 min (to destroy the reverse transcriptase) and cooled at 5 °C for 5 min. After reverse transcription, 78 µl of Mix B was added to each sample. Upstream primer (1.0 µl, to give a final concentration of 0.15 µM) was added and samples were incubated at 95 °C for 1 min (to denature the cDNA). PCR was performed (95 °C for 1 min and 60 °C for 1 min) for 35 cycles.

E.4 Detection of PCR amplification products by agarose gel electrophoresis

After PCR the amplified DNA products were extracted. Chloroform (100 µl) was added to each tube, shaken and the aqueous phase collected. To 50 µl of aqueous phase, a volume of 100 µl of ice cold absolute ethanol was added. Samples were incubated at -20 °C (2 h) and DNA collected by centrifugation (14 000 rpm; 5 min). The supernatant was decanted and the precipitate dried in an oven (44 °C; 15 min). The DNA was resuspended in 10 µl 0.45 M Tris-Borate, 0.01 M EDTA, pH 8.0 buffer (TBE). A 3% agarose gel was made by melting 1.5 g Sigma 3:1 agarose in 10 ml 5x TBE and 40 ml distilled water. The mixture was poured into a gel tray (100 x 76 mm) sealed with adhesive tape. A gel comb had been positioned to allow 11 sample wells to be cast in the gel. The adhesive tape and gel comb were removed when the gel had set and the gel tray transferred to the gel box (GNA 100, Pharmacia). A volume of Sigma loading buffer (2 µl) was added to each sample. This increased the density of the sample. Each sample (10 µl) was loaded into a well and electrophoresis initiated at 65 V, 37 mA and continued until the bands had migrated 75% of the gel (usually 2.5 h). Digests of DNA were also subjected to electrophoresis. These contain DNA fragments of known molecular weight and serve as markers. When electrophoresis was complete, the gel was stained with

100 ml ethidium bromide (1 μg ml^{-1}) overnight. Bands were visualised as orange fluorescence by illumination under UV light.

E.5 Results of detection of viral genomic material by PCR

PCR was performed on genetic material from three viruses. These included two RNA viruses (poliovirus and rotavirus) of health significance to the Drinking Water Inspectorate and a DNA virus for use as a positive control for PCR. In addition, the Perkin Elmer RNA PCR kits supplied a positive control RNA to check the efficiency of the kits. Results of PCR experiments to amplify genetic material from these systems are now reported. Inhibitory effects on PCR amplification by viral extracts were identified.

E.5.1 Perkin Elmer positive control RNA supplied with RNA PCR kits

Both RNA PCR kits (rTth kit and RTase kit) provided a positive RNA control and primers which on amplification should produce a 308 bp DNA duplex which is detectable with ethidium bromide after separation on agarose gel electrophoresis. Initial attempts to amplify the positive control RNA with the rTth kit were not successful at WRc although the kit was functioning correctly on return to Perkin Elmer Cetus. On discussion with Perkin Elmer Cetus the nature of the sterile water was identified as a possible cause of the failure. WRc repeated the RNA PCR amplification of the positive control RNA using molecular biology grade water (Sigma) and an amplified product band was detectable on agarose gel electrophoresis after staining with ethidium bromide.

The RTase RNA PCR kit gave inconsistent results in that the amplified product was detected in approximately half of the experiments.

E.5.2 Attempt to detect poliovirus RNA using PCR

PCR amplification of poliovirus genomic RNA has been performed with the PCR primers, PG1 and PG2, (Table E.1) on two systems:

1. RNA released from poliovirus cultures by heat treatment of the virions immediately prior to PCR. Ten serial dilutions of a poliovirus culture (5 x 10^8 pfu per ml) were made and 5 μl portions used in Mix A (Appendix E.3.2) with the rTth RNA PCR kit.

2. RNA extracted chemically and purified (Appendix E.3.1) from 0.5 ml poliovirus culture (5 x 10^8 pfu per ml) prior to PCR.

In both cases the rTth RNA PCR kit was used since high temperatures were required to eliminate secondary structure within the poliovirus genomic RNA. Although molecular biology grade water was used, no amplified DNA was detected by agarose gel eletrophoresis.

E.5.3 Attempt to detect rotavirus RNA using PCR

RNA was extracted from rotavirus cultures (Appendix E.3.1) and then amplified by PCR using the RTase RNA PCR kit and the primers, G1 and G2 (Table E.2). No amplification products were detectable on gel electrophoresis. Two further attempts at RNA extraction and PCR amplification were performed. Successful amplification by PCR was not achieved.

E.5.4 PCR amplification of adenovirus DNA

The genetic material of adenovirus is DNA rather than RNA. Adenovirus may therefore be detectable by direct PCR of the DNA, i.e. without reverse transcription. PCR amplification of adenovirus DNA with the PCR primers A1 and A2 (Table E.3) yielded a single strongly-labelled band on agarose gel electrophoresis. Successful amplification of adenovirus DNA was reproducible.

E.5.5 Studies of inhibition of PCR

The presence of extracted rotavirus RNA and its primers (G1 and G2) was found to inhibit PCR amplification of adenovirus DNA by the primers, A1 and A2. Thus PCR amplification of adenovirus DNA alone yielded a strong band on electrophoresis. In the presence of rotavirus RNA extract no band from adenovirus was observed.

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