DETECTION OF ENTEROPATHOGENS BY PCR

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ABSTRACT

The widespread problem of contaminated water by enteric microorganisms necessitates the need to develop a rapid protocol to detect pathogens in water bodies. Usual methods like plating, biochemical tests and use of DNA probes are time consuming which is a limiting factor especially in epidemic situations. Moreover, some cells can exist in a virulent viable but non-culturable state making detection very difficult by plating method. This paper describes a rapid method to detect the enteropathogens, *E.coli*, *Salmonella* and *Vibrio* and enteric viruses from water samples by Polymerase Chain Reaction. *E.coli* was specially chosen as it is an indicator of fecal contamination. The sensitivity of *E.coli* detection was improved to 10 cells to keep in tune with the WHO guidelines. The presence of *E.coli* would also indicate the probable presence of other pathogens.

KEY WORDS : PCR, enteropathogens, monitoring, gene probes.

INTRODUCTION

Decreasing availability of clean water is a major concern all over the world especially in developing countries. The WHO report in 1996 states that the developing world faces a high risk from cholera and typhoid infections (1). The main reason being lack of sanitation and availability of clean potable water. A delegation appointed by UNSECO revealed that 3.3 billion people die annually from diseases caused by water borne pathogens (2). The "Global Water Partnership" was launched in August 1996 with the sole aim of providing safe water by helping countries in the sustainable management of their water resources (3). The gravity of this situation prompted various research groups to develop a rapid method to detect enteropathogens.

The amplification of specific regions of DNA from complex target sequences by the Polymerase Chain Reaction (PCR) has become a standard molecular tool. It has a variety of uses including cDNA cloning, mutagenesis, DNA sequencing, gene walking, and in situ hybridization (4). We have

used this molecular technique to identify enteropathogens *E.coli*, *Salmonella* and *Vibrio* which are the most common contaminants in water bodies. The method has been tested for the entero-pathogen isolates from India, Bangladesh, USA.

DETECTION OF ENTERIC PATHOGENS

To facilitate detection of microorganisms immuno-detection systems were developed in the early seventies. This was an improvement as the identification protocols by biochemical tests. The sensitivity of detection was further improved by the development of gene probes. DNA probes have been developed to detect *Entamoeba histolytica*, the causative agent for amoebiasis (P. Padmanabhan et. al., 1997 personal communication). Detection of *Salmonella* by gene probes was also established by a number of workers (5). The gene probe technology finds a limitation in detection of non-cultural but viable microorganisms. The prime example of this being *Salmonella* sps. This drawback can be overcome by using the PCR technology which minimizes the time of detection technology which minimizes the time of detection and has the ability to amplify target

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sequences from crude DNA preparations.

Another lethal contaminant in water samples is infection by virus particles which are very difficult to detect. Cell culture methods have now been replaced with molecular biology techniques. This is mainly helpful to detect viruses like hepatitis A & E, Caliciviruses, rotaviruses and astroviruses which do not grow or grow with great difficulty in cell cultures. Radioimmuno assay, enzyme-linked immunosorbent assay lack the degree of sensitivity required to detect the low quantities of virus expected in samples from environmental sources. Hybridization assays with cDNA or ssRNA probes have been replaced by PCR for DNA virus or reverse transcription followed by PCR (RT-PCR) for RNA viruses. PCR techniques have been applied for the detection of enteric virus in widely different environmental samples (6).

The PCR reaction has also been successfully demonstrated in detection of Hepatitis E. Virus (HEV) from waste water. HEV was concentrated from sewage samples. In acidic conditions $MgCl_2$ was added to a final concentration of 1200 mg/ml and filtered. Elution of virus particles from the filters was performed with urea (1.5 M) -arginine (0.02-0.0083 M) phosphate buffer (VAPB). Reconcentration of this elute was done after precipitation with $MgCl_2$ by suspending it in McIlvaines buffer (7). PCR was also used to detect Rota virus in the Madras epidemic in 1992/93 (8).

DETECTION OF ENTEROPATHOGENS *E. COLI*, *SALMONELLA* & *VIBRIO*

PCR protocols also have been successfully extended for detection of bacterial pathogens. In this report various amplification strategies were worked out to assess the flexibility of various physico-chemical parameters keeping the target specificity of the primers. This will help in designing a simultaneous detection of various target pathogens in a single reaction i.e. development of multiplex PCR protocol

There are a number of factors that can affect the specificity of the amplification reaction, like primer concentration, $MgCl_2$ concentration, annealing temperature, and time of the three reactions steps viz., denaturation, annealing and extension. The PCR protocol was optimized first for concentration of primers. The concentration was

varied from 0.5 μM to 2 μM and it was observed that the best results were obtained when the primer concentration was 0.5-1 μM which was used further for subsequent experiments. Higher concentration resulted in non-specific annealing with the temperature used for optimization viz. 50°C-68°C (data not shown).

PRIMERS

The 24 mer primers used to detect *E. coli* amplified a 309 bp segment of the coding region from lam B gene of *E. coli*. These primers were designated BL-4910 (5'- CTG ATC GAA TGG CTG CCA GGC TCC) and BR-5219 (5'- CAA CCA GAC GAT AGT TAT CAC GCA) and are specific for fecal *E. coli* (9). The pho E sequence of *Salmonella typhimurium* was used to develop primers SF5 (5'- AGC GCC GCG GTA CGG GCG ATA AA) and ST8 (5'- ATC ATC GTC ATT AAT GCC TAA GGT) which are based on the 9 sequences encoding the fifth and eighth surface-exposed regions of pho E. They amplify a 365 bp DNA fragment from target *Salmonella* (10). For detection of *Vibrio*, the target sequence chosen was ogawa serotype. The primers used (5'- CAG AGC TTG ATG CTA TTG AGT CAA AG) and (5'- TAA ACT CAG GAT GTT CAG CGG ATG) amplify a 765 bp fragment (11). All primers used were purchased from Integrated DNA Technologies, Inc. (IDT) USA.

The concentration of $MgCl_2$ was varied from 0.5 mM to 6 mM. Optimum results were obtained at a concentration of 1.5-3 mM. Lower concentrations reduce the yield while higher concentration increases the amplification of non-specific products.

The concentration of the template also plays a crucial role in the PCR reaction. Low concentrations of template increase non-specific product formation and also cause the "primer dimer" artifact. Sensitivity of the PCR reaction plays a crucial role if we need to detect pathogens from environmental sources. We have achieved a sensitivity of detection of 103 cells of *Salmonella* and *Vibrio*. The sensitivity of *E. coli* was improved to detection of 10 cells. The importance of this being that the presence of *E. coli* indicate the presence of fecal contamination and the possible presence of other pathogens. The dose of *Salmonella* required to cause disease is known to be 10^5 - 10^6 . Fig. 1 shows the detection of *E. coli*, *Salmonella* and *Vibrio* by PCR.

Amplification of enteropathogens *E.coli*, *Salmonella* and *Vibrio* by PCR

Lane 1 : 1 kb ladder
 Lane 2 : lamB product (309bp)
 Lane 3 : phoE product (365bp)
 Lane 4 : ogawa product (765bp)
 Lane 5 : *E. coli* - *Salmonella* duplex
 Lane 6 : *E. coli* - *Vibrio* duplex
 Lane 7 : *Vibrio* - *Salmonella* duplex

The PCR results indicate that product formation occurs in a varied range of conditions namely primer concentration $MgCl_2$ concentration and annealing temperature. This flexibility can be manipulated to design a multiplex PCR protocol which would use all the three primers and template in a single reaction.

CONCLUSION

Optimization of the various factors involved in the PCR reaction was carried out with the aim that the single organism specific PCR could be extended to the multiplex PCR where in all three pathogens can be detected in a single reaction. This would not only save valuable time but would be more cost effective.

The utility of this method would be in determining the potability of groundwater and drinking water supplies. Before it can be used for estimating public health safety more work has to be done and experiments are in progress to optimize the PCR protocol for the three pathogens directly from environmental water samples.

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