Development of PCR protocol for detection of *Escherichia coli* in drinking water

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Abstract

Escherichia coli is a pathogenic microorganism that may cause severe gastrointestinal illness in humans. This pathogen may be transmitted in a variety of ways, including food and water. As with most waterborne pathogens *E. coli* is difficult to detect and enumerate with accuracy in drinking waters due to methodological limitations. The aim of this study was to develop a PCR protocol for the detection of *E. coli O157:H7* and *E. coli* virulence gene SLT-I (Shiga like toxin) in drinking water using a double enrichment step. This method is comprised of bacterial DNA purification using Genomic DNA extraction kit followed by PCR detection. The PCR optimization was done with *E. coli O157:H7* strain EDL 933 (ATCC 43895). The oligonucleotide primers Rfb and SLT-I were used for targeting O157 and SLT-I genes respectively. The specific PCR product of these primers were obtained at 292 bp and 210 bp for Rfb and SLT-I respectively, which were visualized by gel electrophoresis and ethidium bromide staining. In spiked water samples, PCR results showed high sensitivity (<100 CFU/L) for *Escherichia coli*. The results obtained showed that the developed protocol would be utilized as a routine analysis for monitoring drinking water contamination. Furthermore, the simple and rapid protocol of the proposed technique provides results at a fraction of time required by the traditional culture techniques (24 hours compared to 2–6 days).
Keywords: Escherichia coli, PCR, drinking water, O157:H7, SLT-I, Rfb, enrichment.

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1 Introduction

Water related diseases are the major cause of human illness in developing world, due to poor and unhygienic living conditions. The per capita income is very low and people cannot afford to purchase bottled water. The consumption of untreated and contaminated water make humans vulnerable to major diseases like haemorrahgic colitis, haemolytic uraemic syndrome or thrombotic thrombocytopenic purpura. These diseases are related to *Escherichia coli* O157:H7 and enterohaemorrahgic *Escherichia coli* strains. These organisms can produce potent cytotoxins, similar to shiga toxins (like shiga like toxins I and II). Production of shiga toxin is the virulence trait of some *Escherichia coli* strains and some studies revealed that environmental waters are the major reservoir of infectious *Escherichia coli* (Bonetta *et al.* [1]).

Pakistan is a South East Asian developing country and consumption of contaminated waters is a very common phenomena because lack of drinking water treatment facilities and mismanagement of the existing ones. Although it is blessed with adequate amount of ground and surface water, with about 150 million acre feet (MAF) water available annually. Per capita water availability is decreasing in Pakistan from 5,000 cubic meters to 1,100 cubic meter, the reasons for this decline are many (SCEA [2]). Primary factor is the public's concern on achieving the better life standards resulted in mass migrations from rural to urban areas as a result of rapid industrialization, common phenomena in most developing countries. The sprawling industrial areas and urban vicinities followed many problems in the midst of them water availability and sanitation are feverish issues. Almost 60% of the population in Pakistan gets their drinking water share from groundwater aquifers. This percentage varies among different provinces and also amid different cities within the provincial territories [3]. According to a media report more than 80% of reported cases in hospitals of Rawalpindi were due to water borne diseases. In 1993 an epidemic of hepatitis also occurred in the twin cities resulted in 4000 cases and the reason was contaminated water consumption among patients. The diarrheal episodes were prevalent in the twin cities and the morbidity rates among children under 5 years of age are higher due to bacteriological infections according to a report. Diarrhea ranked priority disease causing human mortalities and its causative agents percolates from fecal contamination of water bodies. Major urban cities are not provided with waste water treatment plants and almost 60–80% of urban waste water gained entry into fresh water bodies [3]. The rivers are considered to be the major recharge zone of ground water in cities. As a result of trans movement of chemicals the sewage and industrial effluent percolates into groundwater (Jyoti *et al.* [4]).

The methods which are recently used by some laboratories to test the bacteriological contamination of drinking water take 2–4 days. These methods include Most Probable Number (MPN), Membrane Filtration Technique (MFT) and other biochemical tests for isolation and identification of major water contaminants like *Escherichia coli*, *Salmonella* and *Shigella*. Sometimes they do not give accurate results due to the presence of non culturable strains. These

strains when gained entry into a host they become viable but are not culturable on a media. A rapid monitoring system is coveted for drinking water supplies in order to provide safe and healthy water to consumers. The Polymerase Chain Reaction (PCR) is a molecular method, which can be used for detection of pathogenic microbes from food, water and other environmental samples. It can amplify a very small amount of microbial DNA present in sample to thousand of multiple copies and provide information about prevalence of bacterial pathogens in the sampling area (Duarte *et al.* [5]). This method may facilitate epidemiological surveillance of water and edible products for human consumption to determine the risk of acute diarrheal disease at any single time in any densely populated geographical region. However the sensitivity of PCR can be influenced by physical dilutions of the samples and this can be substantiated by coupling PCR with the enrichment step. Enrichment can lower the detection limit and dilute the inhibitory substances present in the sample and may also reduce the possibility of detecting dead cells (Moganedi *et al.* [6]). The current study aimed at developing a PCR protocol coupled with enrichment step in order to monitor the microbial quality of drinking water within 24 hours. It would be helpful and provides a framework to government institutions for designing rapid and accurate monitoring programme for drinking water quality.

2 Material and methods

2.1 Bacterial strains and culture media

The pure culture of *Escherichia coli* O157:H7 strain EDL 933 (ATCC 43895) has been obtained from the American type culture collection center (ATCC), the cultures were handled as per manufacturer's instructions. The strains were streaked on nutrient agar (Merck) slants and incubated at 37^{o}C.

2.2 Primers and molecular markers

The primer pairs used for *Escherichia coli* O157:H7 detection were Rfb-F, Rfb-R and SLT-I F, SLT-I R. This set of primers helps in revealing the serotype O157 of *Escherichia coli* and also its virulence trait shiga like toxin (SLT) production. This would be helpful in serotype identification and toxin profiling of the specie prevalent in the region. The primers were procured from IDT; the detailed primers are given in table 1. All primer sequences were compared against each other and homology searches performed against the gene bank database for sequence similarity using the BLAST (Basic logic alignment search tool) programme http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM. The computer generated data revealed that both primer pairs showed significant affinities for their target genes only.

The DNA ladders (Norgen) of 50 and 100 bp have been used for comparing the extracted DNA of the microbe and to determine the PCR product size.

Table 1: Targeted genes of *Escherichia coli* and their primer sequence details.

Target Gene	Primer	Sequence	Product size (bp)	Tm (°C)	Length of primer (bp)	GC content (%)
Escherichia coli						
O157	Rfb F	GTGTCCATTTATACGGACATCCATG	292	55.6	25	44
	Rfb R	CCTATAACGTCATGCCAATATTGCC		56.1	25	44
SLT-I	SLT-I F	TGTAACTGGAAAGGTGGAGTATAC	210	53.9	24	41.6
	SLT-I R	GCTATTCTGAGTCAACGAAAAATAAC		52.7	26	34.6

2.3 Template preparation

Two methods have been used for extraction of genomic DNA of microbes. Phenol chloroform method and PrepEase genomic DNA isolation kit (Affymetrix, product # 78850).

2.3.1 Phenol chloroform method

As per the method described by Hu *et al.* [7]. The 1.5 ml suspension of bacterial culture was centrifuged for 3 minutes at 12,000rpm supernatant was discarded and cell pellet was used. The cell pellet was well mixed with 200µl of lysis buffer pH 7-8 (40Mm tris acetate pH 7.8, 1Mm EDTA, 20Mm sodium acetate, 1% SDS). RNA's is added if required for removing RNA from sample. After this step 55µl of 5M NaCl solution was added in order to remove the cell debris and proteins. The mixture was centrifuged at 12,000 rpm for 10min at 4°C. After centrifugation supernatant was transferred into a new vial and equivalent volume of chloroform was added into it. The eppendorf was inverted 5–10 times; a milky solution was formed and then centrifuged at 12,000 rpm for 3 minutes. The extracted supernatant was used and 100% ethanol is added into the vial. This solution put into -20°C freezer for one hour, DNA was precipitated. The solution centrifuged at 12,000 rpm for 5 minutes the supernatant discarded and DNA pellet was further washed twice with 70% ethanol followed by centrifugation at 12,000 rpm for 3 minutes. It was than dried in incubator at 37°C for 15 minutes. The pellet was dissolved into 50µl of 1x TE buffer (1M Tris HCl pH 8.0, 0.25M EDTA) and store at -20°C (Hu *et al.* [7]).

2.3.2 Genomic DNA isolation kit

Genomic DNA isolation kit (Affymetrix, product # 78850) was used for DNA extraction from bacterial cultures. Fresh culture of 30mg wet bacterial pellet was used for extraction of DNA. The sample was lysed by adding 0.24ml of homogenization buffer and mixed well on vortex mixer. The 0.2ml of chloroform/Isoamyl alcohol (24:1) and 0.8ml of protein precipitation buffer was added into the lysate. The mixture was vortexed followed by centrifugation at

13,000 rpm for 4 minutes. After centrifugation the 0.88ml of supernatant was transferred into new 1.5ml eppendorf containing 0.62ml of iso-propanol, the genomic DNA precipitates as white fibre like strings. This solution was mixed thoroughly and centrifuged at 13,000 rpm for 4 minutes. After centrifugation DNA pellet obtained and supernatant was discarded. The pellet was washed once with 1 ml of 70% ethanol by vortexing followed by centrifugation at 13,000 rpm for 2 minutes. The supernatant was aspirated without disturbing DNA pellet. The pellet was dried for 5 minutes at 37^{o}C and resuspended in 150µl of DNA resuspension buffer. Vortex to dissolve the DNA pellet and store at 4^{o}C.

2.4 PCR optimization

PCR was optimized for *Escherichia coli* genes O157 and SLT-1 with pure culture of *E. coli* (ATCC 43895). It was optimized by adjusting different parameters such as $MgCl_2$ concentration, annealing temperature and primer concentration. The concentration of $MgCl_2$ was varied from 1-3mM with increment of 0.5mM, after optimization of $MgCl_2$ concentration the other parameter optimized was annealing temperature. It varied between 48–61^{o}C by increasing 1^{o}C at a time. The primer concentration was checked at 3 points (20pM, 2µM and 10µM) described previously in literature. Each parameter in the listed order was optimized while other kept constant. The PCR reaction mixture for the reference PCR protocol consisted of 10 x PCR buffer (50mM KCl, 10mM Tris-HCl, 0.1% Triton X), 5U Taq DNA Polymerases (Finnzymes), 1Mm $MgCl_2$ (Bioron), 2µM each primer (IDT), and 200µM each of dNTPs. The PCR cycling conditions were: Denaturation at 94^{o}C for 2 min, 35 cycles of primer annealing consisted of 94^{o}C, 55.5^{o}C and 72^{o}C each for 30 seconds in each cycle followed by extension at 72^{o}C for 5min. Amplification was performed in EG9600 PCR gradient thermal cycler (Extra Gene USA).

2.5 Detection limit of PCR

The sensitivity of PCR protocol for detection of *Escherichia coli* in drinking water was determined. The drinking water samples were spiked with known volume of microbial culture and 10 fold serial dilutions were made and incubated for 4 hours at 37^{o}C. The viable count of cell suspension in each dilution was counted by plating 1ml on Luria Bertani Agar (LBA) plates. By calculating the cell numbers from three replicas, plate's average count was determined. Genomic DNA was extracted using phenol chloroform method by taking 1ml from each suspension (Hu *et al*. [7]). The DNA was used for PCR by following the optimized conditions. To analyze the amplified PCR product by electrophoresis (Mupid one), 10µl aliquot of the PCR product was loaded on 1% agarose gel stained with ethidium bromide and run in 1x TBE buffer at 70V/cm for 45 minutes.

2.6 Protocol for sample analysis

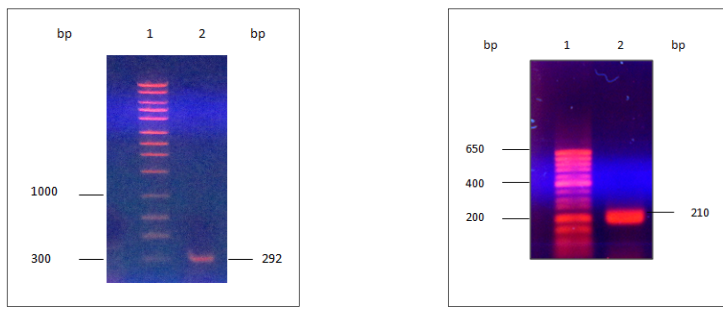
One liter of water sample spiked with *Escherichia coli* O157:H7 strain was concentrated by filtering through 0.45µm pore size nitrocellulose membrane. The

filter was vortex in nutrient broth and the broth containing the filter incubated at 37°C for 4 hours. Following incubation 2ml of broth was centrifuged at 5000 rpm for 10 minutes to recover the bacteria. The obtained bacterial pellet was subject to DNA extraction and used for PCR detection. The primers SLT-I and Rfb were used for targeting the O157 and SLT-I gene of *Escherichia coli.*

3 Results

3.1 PCR optimization

Specific and sensitive amplification of O157 and SLT-I genes by PCR is dependent on several factors like Mg^{2+} ions concentration, primers concentration and annealing temperatures which are also dependant on melting temperatures (Tm) of respective primers. The Taq polymerases also plays important role in amplification and it requires divalent ions such as Mg^{2+} for activity and it acts as a co factor and forms complexes with dNTPs which polymerase recognize for activity. Mg^{2+} also effect the primer annealing, strand dissociation temperatures of template DNA and primers and formation of primer-dimer artifacts. The best results for amplification occurred with 2mM $MgCl_2$ with 2µM primer concentration. When the amount of Mg ions and primer concentration increased the primers–dimer structure appeared and the lower concentration of both parameters gives very light intensity bands, which are not clearly visible. No amplification occurred with 1mM $MgCl_2$ and 20pM primer concentration. The low annealing temperatures and high primer concentration allow mis-priming whose product competes with the target sequence of primers. The O157 gene gives best yield (product size 292bp: Figure 1(a)) at 55.5°C and SLT-I (product size 210bp: Figure 1(b)) amplified at 54.2°C with 2mM $MgCl_2$ and 2µM primer concentration. The optimized PCR reaction protocol in 10µl volume is as follows: 1µ-10x PCR buffer, 0.4µl $MgCl_2$ (2Mm), 2µl each Forward and reverse



(a) (b)

Figure 1: Agarose gel of PCR product amplified from *E. coli* O157:H7 strain (ATCC43895). (a) Lane 1 shows 1kb DNA ladder, lane 2: O157 gene (292bp) amplified with Rfb F and R primers. (b) Lane 1 is 50bp DNA ladder and lane 2 SLT-I gene (210bp) amplified with SLT-I F and R primers.

primers(2µM), 0.8µl dNTPs (200µM), 0.3µl 5U Taq Polymerase, 1µl template DNA and PCR water was added to adjust the total volume up to 10µl. the cycling conditions were: Denaturation at 94°C for 2 min, 35 cycles of primer annealing consisted of 94°C, 55.5°C for Rfb and 54.2°C for SLT-I and 72°C each for 30 seconds in each cycle followed by extension at 72°C for 5min.

3.2 Detection limit of PCR

The spiked water samples when plated on LBA the dilution from 10–10^{-5} have too numerous to count number of colony forming units. The dilutions from 10^{-6}–10^{-10} have countable numbers, which gave us an idea about the sensitivity of PCR. The detection limit was determined to be as low as 3 cfu/ml. The PCR gives best intensity bands at each dilution as shown in Figure 2(a) and (b) for O157 and SLT-I genes respectively. The difference in band intensities of each homologous PCR product showed variation of cell densities within the samples. The sensitivity can be further increased by enhancing the enrichment time from 4 hours to 6 hours or 8 hours.

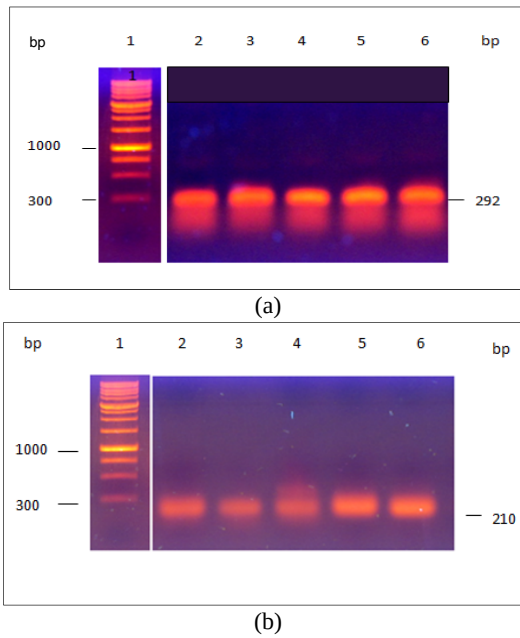


Figure 2: Agarose gel showing detection sensitivity of PCR, at different cfu/ml in spiked water samples. Lane 1: 1kb DNA ladder and lanes 2–6 are depicting 1.1x10^2cfu/ml, 2x10^2cfu/ml, 2.32x10^2cfu/ml, 3.3x10^2cfu/ml and 4.1x10^2cfu/ml in both figures. Fig (a) shows results for O157 gene and Fig (b) for SLT-I gene.

3.3 Protocol for sample analysis

Using the optimized PCR protocol analysis of spiked water samples for *Escherichia coli* was done, as shown in figure 5 for Rfb and SLT-I genes. A preliminary enrichment step before amplification is necessary because the molecular methods have lower detection limit. *Escherichia coli* and other water borne pathogens if present in water sample as low as 1-3cfu/ml poses serious health risk to human beings. The sample filtration step is coupled with enrichment because real water samples have very low amount of pathogenic microbes and can be captured on membrane filters for harvesting. This step did not interfere with the results because the products of PCR were obtained and gave sharp bands compared to the analysis without filtration during sensitivity detection.

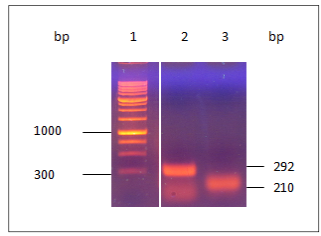


Figure 3: Agarose gel showing PCR amplification products of O157 and SLT-1 genes in drinking water samples. Lane 1: 1kb DNA ladder, lane 2: 0157 gene (product size 292 bp) and lane 3: SLT-I gene (product size 210bp).

4 Discussion

The current study was conducted to develop a protocol for microbial analysis of drinking water and also to determine the prevalence of O157 and SLT-I genes in the water. The Rfb and SLT-I primers with Tm's of 56^{o}C and 54^{o}C were amplified at 55.5^{o}C and 54.2^{o}C respectively. By adjusting the parameters like Magnesium ion concentration, annealing temperatures, primer concentration and template concentration, very sharp and intense bands of PCR amplification were obtained at their respective product sizes. This indicated that all these parameters have effects on activity of Taq DNA polymerases (enzyme) which requires Mg^{2+} ions for its activity. The annealing temperatures ranging between 20-85^{o}C gave best amplification results because enzyme activity is greatest between these two orders of magnitude. The concentration of Taq polymerase was also very important if its concentration is very high non specific background products may accumulate and insufficient desired product may be obtained if it's in low amounts (Innis *et al.* [8]). After amplification samples were separated in agarose gel electrophoresis, 1% agarose gel was used stained with ethidium bromide solution. DNA bands visualized under UV light and images were captured by high resolution camera.

The monitoring of drinking water for presence of *Escherichia coli* is important in order to ensure the public health. *Escherichia coli* O157:H7 usually associated with outbreaks and sporadic cases of human diseases, its infection symptoms are intestinal problems such as abdominal pain and bloody or non-bloody diarrhoea (Tsen and Jian [9]). The high throughput PCR protocol developed by targeting SLT-I and O157 genes of *Escherichia coli* provide a powerful supplement to conventional methods for more accurate risk assessment and monitoring of pathogenic bacteria in drinking water. Shiga like toxins are associated with many shiga toxins producing *E coli* infections in humans and toxin profiling and serotype identification is necessary for their identification from environmental samples. The traditional methods are very time consuming and PCR provides advantage by detecting this virulence trait (Parma *et al.* [10]). The RfbE gene encoding the enzyme involved in biosynthesis of O157 antigen has been identified and this gene is divergent from rfb loci encoding for O antigen. The Rfb cannot differentiate the O157:H7 from other O157 isolates and for future work Flich primers would be used to differentiate H7 antigen and it provides a complete profile of O157:H7 antigen (Bonetta *et al.* [1]). This type of protocols may further be improved by multiplex PCR system to analyze more than one pathogen in a single reaction and provide complete microbiological risk evaluation of water.

The current protocol is combined with a 4 hour enrichment step because drinking water samples contain a very low number of pathogenic microbes. The enrichment gives high sensitivity to PCR assay and its detection limit is as low as <100 cfu/ml. The advantages of using PCR along with enrichment and filtration step for drinking water analysis are: its low detection limit, specificity of the analysis by targeting specified genes of the microbes and rapidity. It also allows the effective assessment of filtration and treatment units by allowing pre and post treatment analysis of water. This protocol may be used on regular basis for monitoring of drinking water samples within 24 hours and this method proved to be efficient for detecting low levels of pathogens as compared to traditional methods which cannot detect non culturable and non viable microbes and are also very time consuming (2–4 days).

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