KATHMANDU UNIVERSITY JOURNAL OF SCIENCE, ENGINEERING AND TECHNOLOGY
VOL. 5, No. II, SEPTEMBER, 2009, pp 130- 136.

MOLECULAR CONFIRMATION OF THE PRESENCE OF COLIFORMS IN DRINKING WATER USING POLYMERASE CHAIN REACTION

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Received 2 July, 2009; Revised 7 September, 2009

ABSTRACT: Coliform bacteria include organisms like *Escherichia coli, Enterobacter, Klebsiella and Citrobacter* are gram negative, facultative anaerobic, non-sporulating and lactose fermenting organisms. *E.coli* is used as the indicator organism for detection of fecal contamination of water. Conventional methods for the detection of these Coliforms in water include microbial culture assays in lactose containing media and also enzyme based assays for the detection of β-galactosidase using chromogenic substrates like *ortho*-nitrophenyl-β-D-galactopyranoside. These methods are cumbersome with limited specificity. DNA based molecular techniques like Polymerase chain reaction is recommended since it is highly specific. In this paper, we report the use of PCR for the amplification of the Lac Z (β-galactosidase encoding) gene to confirm the isolation of Coliform bacteria from drinking water samples. The method could be an effective epidemiological tool to pin point the source to contain outbreaks of waterborne disease episodes.

Key words: Potable water, Coliforms, Polymerase Chain reaction, β-Galactosidase

INTRODUCTION

The coliform bacteria are one of the common contaminants present in drinking water. Therefore, detection of coliforms as indicators of human fecal contamination is very important to protect public health. Coliforms belong to the family Enterobacteriaceae and they are facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35°C (APHA,1998). *E. coli* is recognized as a good indicator of faecal contamination. It is identified as the only species in the coliform group found exclusively in the intestinal tract of humans and other warm-blooded animals and subsequently excreted in large numbers in feces, approximately 10^9 per gram (Geldreich, 1983). According to the WHO guidelines for microbial quality of drinking water, in all water intended for drinking, *E.coli* or thermotolerant Coliform bacteria must not be detectable in any 100 ml sample. The conventional methods of detection of coliforms are by the most probable number, MPN method. Further microbial and biochemical analysis will have to be performed for the definite identification of the presence of organisms. Some assays rely on the enzyme β-D-galactosidase

activity of the strains (Edberg, and Kontnick, 1986). These methods have the disadvantages of being time consuming and yielding false positive results (Kampher, 2008, Olstadt, 2007).

In the present, study we have confirmed the presence of coliforms in different sources of potable water by using PCR for the specific amplification of the Lac Z gene that encodes the β-D-galactosidase enzyme. This gene was selected since it is the basis for most of the assays of Coliform detection.

MATERIALS AND METHODS

Sample Collection

Drinking water samples from 8 different sources was included for assay. 5 samples were bottled water obtained from commercial supplier, 2 samples from water purifiers that employ RO –UV treatment and 1 sample was municipal water. Samples were collected for bacteriological (total coliform) analysis in 300 ml capacity autoclaved glass containers having polypropylene lids in duplicates using aseptic techniques. The water samples were immediately taken up for microbial analysis. The water was sampled from same sources and analyzed second time after one month.

MPN Analysis: The MPN tests were performed according to the standard protocols (APHA, 1998). Multiple Tube Techniques utilize selective and differential liquid media into which multiple aliquots of serial dilutions are inoculated. The technique involves three successive steps, namely, presumptive test, confirmed test and completed test.

In the presumptive test, 3 sets of tubes with each set containing five tubes were inoculated with a ten-fold difference in inoculum volume. Each set is inoculated with 0.1 ml, 1 ml and 10 ml per tube. The medium used was Phenol red Lactose Broth (Himedia) containing inverted Durham's tubes. After incubation at 37^0C for 48 hrs, the tubes are then examined acid and gas production. Each set is scored for the number of positive tubes and the score of all five sets is then recorded and used with the standard Most Probable Number Table to determine the number of coliforms per 100 ml of water.

The Presumptive Test is the first followed by the Confirmative Test, the Completed Test and the IMViC tests. The Confirmative Test was performed by streaking a positive Presumptive tube onto Eosin Methylene Blue Agar (EMB Agar). This agar contains lactose and the dyes Eosin Y and Methylene Blue. When *E. coli* grows on EMB it ferments so much acid that the two dyes precipitate out in the colony producing a metallic green sheen appearance. A positive Confirmative Test is then the presence of green sheen colonies on EMB streaked from a positive Presumptive Test.

The Completed Test was performed by inoculating a tube of Phenol Red Lactose Broth with a green sheen colony from a positive Confirmative test. Simultaneously, a loop of organism was streaked onto a slant of nutrient agar. Both tubes were incubated at 37 ^{0}C for 48 hrs. The culture on the nutrient agar was analysed by Gram staining.

Biochemical Assays

The predicted positive samples from the above MPN tests were subjected to further biochemical assays. The biochemical tests performed include Indole production, Methyl red, Vogues-Proskauer test, Citrate test, Oxidize production and catalase production. These biochemical tests were performed as per standard Microbiological methods (Cappuccino and Sherman, 2007). Analytical grade chemicals and reagents obtained from Himedia were used for all the tests. An *E.coli* culture obtained from a culture collection center was used as a control for the biochemical tests.

Motility and thermo tolerance

The organisms were inoculated into 5 ml of nutrient broth and incubated overnight at 37^0 C. The motility of the isolated organisms was analysed by the hanging drop method. The organisms were also tested for thermo tolerance by incubating them in broth media at 45^0C.

Isolation of DNA

Single colonies of the isolated bacteria were inoculated into 2 ml of Luria Bertani broth and grown overnight at 37^o C at 150 rpm in a shaker incubator. The cells were pelleted and resuspended in 467µL of TE (10 mM Tris, 1mMEDTA, pH 8.0) buffer. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K was added and incubated for 30 min at 37 ° C. An equal volume of phenol/chloroform was added and mixed well and centrifuged at 12,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube and an equal volume of phenol/chloroform and extracted again. To the upper aqueous phase, 1/10 volume of sodium acetate and 0.6 volumes of isopropanol were added to precipitate the DNA.The DNA was spooled and washed in 70% alcohol. It was then dissolved in 200 µL of TE buffer and stored at 4^o C. DNA concentration was determined by running on a 0.8 % agarose gel.

Polymerase Chain Reaction

The Lac Z gene was amplified using the pair of primers, designated as

1. LZ1 (5'ATGAAAGCTGGCTACAGGAAGGCC3')
2. LZ2 (5'GGTTTATGCAGCAACGAGACGTCA 3') (Bej.Ak *et al..*, 1990, Bej, A.K *et al..*, 1991). LZ1 spans between sequences392-415 of the Lac Z gene while LZ2 spans between the sequences 633-656. They yield an amplified product corresponding to 264 bp.Primers LZ1 and LZ2 and PCR reagents were obtained from Messers Bangalore Genei, Bangalore. A 25 µL of PCR reaction was set up containing Buffer(50mM KCl,50mM Tris-Cl,pH8.9,25mM $MgCl_2$), 200µM each of the dNTPs, 0.5µM of primers, 2.0 Units of Taq polymerase and 200 ng of the template DNA. 25 cycles of amplification were performed in a Corbett Research thermal cycler with the following conditions: Initial denaturation at 95^0C, 3 minutes, denaturation at 94^0C, 1 minute, annealing at 55^0 C for 1 minute, extension at 72^0C for 1 minute and final extension at 72^0C for 2 minutes. The amplified products were visualized on a 2.5 % agarose gel. *E.coli* DNA was used as a positive control and autoclaved de-ionized water was used as a negative control.

RESULTS

MPN analysis

The bottled water samples and the municipal water samples analyzed showed an MPN index that ranged from 2-1600 per 100 ml and 350-1600 per 100 ml respectively. The water samples obtained from the RO-UV water purifier always tested negative with neither change in color of the Phenol red lactose broth medium nor accumulation of gas in the Durham's tubes. The cultures that gave greenish metallic sheen on EMB agar were further subjected to completed tests. A total of 18 isolates that were gram negative rods obtained thus were further analyzed for biochemical tests, motility and thermo tolerance.

Source of Sample	Sample No.	Average MPN index per 100 ml
Bottled water	1	3
	2	1600
	3	71
	4	28
	5	1
Municipal water	6	1600
	7	31
RO-UV treated water	8	0

Table 1: Results of MPN analysis of Set I

Source of Sample	Sample No.	Average MPN index per 100 ml
Bottled water	1	6
	2	< 1.8
	3	< 1.8
	4	28
	5	1
Municipal water	6	1600
	7	31
RO-UV treated water	8	0

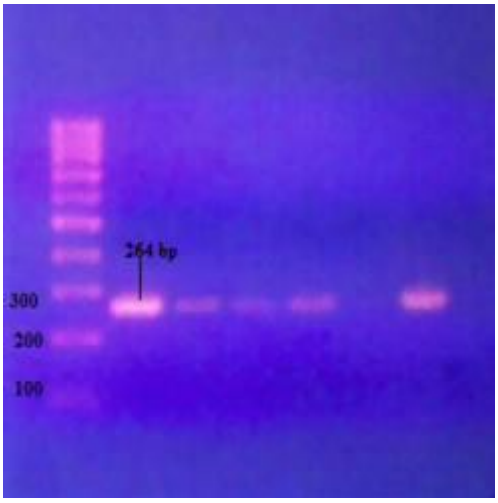
Table 2: Results of MPN analysis of Set II

Biochemical assays, motility and thermo tolerance

Test parameter	Bottled water sample		Municipal water sample	
	Positive (%)	negative (%)	positive (%)	negative (%)
Indole test	43	57	25	75
Methyl red test	71	29	25	75
Vogues Prauskuer test	36	64	75	25
Citrate test	57	43	75	25
Oxidase test	14	86	0	100
Catalase test	100	0	100	0
Motility	100	0	100	0
Thermotolerance	100	0	100	0
Lac Z gene	64	36	100	0

Table 3. Results of various test parameters expressed in Percentage of cultures.

PCR:



PCR amplification of Lac Z gene

Lane 1- 100 bp ladder, lane 2-4 Lac Z amplified from isolated organisms, lane 5- negative control (sterile de-ionized water), Lane 7-positive control-*E.coli*

PCR analysis of 18 isolates obtained after MPN analysis of different water samples revealed that 13 of the organisms were positive for Lac Z gene. They exhibited a clear 264 bp fragment upon amplification. A similar band was found with the positive control *E.coli* DNA that was used and no amplification was observe with negative control in which sterile de-ionized water was used instead of DNA. 5 organisms derived from bottled water and municipal water showed no amplification of Lac Z gene.

DISCUSSION

Conventional methods of coliform detection have limitations, such as long durations of incubation, antagonistic organism interference, lack of specificity and poor detection of slow-growing or viable but non-culturable (VBNC) microorganisms.

Detection of coliforms by molecular methods is highly recommended as these methods allow for very specific and rapid detection. Three molecular-based methods are generally used: the immunological, polymerase chain reaction and in-situ hybridization techniques (Rompré *et al.*, 2002). In the immunological approach, various antibodies against coliform bacteria have been produced, but the application of this technique often showed low antibody specificity. PCR can be used to detect coliform bacteria by means of signal amplification: DNA sequence coding for the lac Z gene (beta-galactosidase gene) and the uidA gene (beta-D glucuronidase gene) has been used to detect total coliforms and *E. coli*, respectively (Locas *et al.*, 2008). dct A, dcuB, frdA, dcuS and dcuR genes of the *E. coli* was adapted for use as a non-cultivation-based molecular approach for detection of *E. coli* populations from water samples without the need for pure and identified cultures (Abo-Amer *et al.*, 2008) . But none of these molecular methods have been standardized for routine usage.

CONCLUSION

PCR is a specific and reliable method for non ambiguous detection of coliforms and hence can be popularized for routine laboratory assay. In the wake of detection of Coliforms in the bottled water samples as well as in municipal water, more stringent regulations for the suppliers of bottled water and routine inspections by health officials is recommended from the perspective of minimizing water borne diseases.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Management of PESIT for their support in carrying out this work.

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