Comparison of PCR Technique with MPN Method in Identification of Coliform Bacteria in Water

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Abstract

Microbial pathogens are among factors posing significant threats to hygiene issues for water and wastewater. Thus indicator bacteria are used for detection of relative risk of fecal contamination and potential presence of pathogens in water. However, indicator organisms bear some disadvantages decreasing their effectiveness as ideal indicators of microbial pathogens in water. In addition, conventional methods for detection of these pathogenic viruses, bacteria, protozoa and worms tend to be inaccurate, time consuming and expensive. Polymerase Chain Reaction (PCR) technique has been recommended as a diagnostic technique which has been tested and brought about impressive results. In this paper, following designing primer related to genomic segment of Lac Z it is duplicated using PCR technique in order to isolate bacteria from samples of drinking water. This genomic segment encodes β-galactosidase enzyme in Colifrom bacteria. Then results are compared with multiple-tube fermentation technique by MPN method. Sampling was performed in 18 wells of drinking water in Qom's villages and samples were tested using PCR and MPN techniques. Of the total samples tested, 3 samples responded positively to MPN technique and 8 samples responded positively to PCR technique. Findings suggest higher accuracy in PCR compared to MPN.

Keywords: Drinking water, Polymerase Chain Reaction (PCR), VBNC condition, β-galactosidase

1. Introduction

Coliform bacteria are used as indicator for fecal contamination of water and for determining potential presence of enteric pathogens in order to monitor bacteriologic health of water supply resources (Seema Tharannum et al., 2009; Bej et al. 1990). Conventional methods used for detection of Coliform bacteria are based on optional culture media. Such media allow gram negative bacteria growth and are able specifically to identify lactose consuming bacteria which can grow in EMB, m-Endo, MacConkey or brilliant green culture media (Sherfi et al., 2007; Rompré et al., 2002). Using these culture media and heating at 37 °C, total Coliforms including members of Escherichia, Enterobacter, Citrobacter, Kelebsiella genera are counted. Fecal Coliform bacteria, especially E.coli are counted in increased heating temperature up to 44.5 °C. Since E.coli is mainly seen in human feces, it is used as fecal contamination indicator of water and it is considered as a suitable indicator for monitoring water resources and potential presence of enteric

pathogens in water (Bej et al., 1991; Tantawiwat et al., 2005)

Here some problems are raised regarding culture methods used for routine bacteriological monitoring of water resources including keeping alive bacteria from sample collection to culture time, lack of growth in viable but non-culturable (VBNC) bacteria such as bacteria shocked due to addition of chemicals to water, time required for detection and confirmation of enteric bacteria (lasting often 48-72 h), and lack of a exclusive feature for identification of real fecal Coliforms (E.coli) in conventional laboratory methods (Bej et al., 1991; Byrd et al., 1991; Rompré et al., 2002). Additionally, the other reason for necessity of new approaches to microbial monitoring of drinking water resources is WHO guidance (2011) which points out E.coli bacteria or heat resistant Coliform bacteria should not be observed in 100 ml of samples in any samples of drinking water. Thus according to method standard book, it has been mentioned that the accuracy of MPN technique in detection of the Coliform bacteria is relatively low (Apha, 2005). In addition, studies by microbial ecologists suggest very high presence of bacteria capable of entering into VBNC condition in different environmental samples (Byrd et al., 1991). VBNC bacteria are a major concern in public health risk assessments since most pathogenic bacteria such as Vibrio cholerae, Mycobacterium tuberculosis, Campylobacter jejuni, Helicobacter pylori, Vibrio vulnificus and Escherichia coli have been reported in connection with entry into the VBNC condition. This group of bacteria is capable of growth and proliferation and infectivity upon their entrance to animal hosts (Byrd et al., 1991; Shahian et al., 2009). Therefore, in order to prevent mentioned problems in detecting Coliform bacteria, genetic methods without need for culturing have been used which are based on amplification of specific sequences of DNA with PCR technique and replicated genome identification. Advantages of PCR system include its high sensitivity (which is capable of identification of even one cell in 100 ml of water sample), method specificity for the target microorganisms, high speed testing from samples' collection to completion of their analysis (less than 6 h), and the ability to detect multiple bacteria simultaneously (including general indicator species and a series of specific target pathogens) (Bej et al., 1990; Bej et al., 1991; Hongying Fan et al., 2008). PCR requires a sample molecule, that is, DNA or RNA which is to be duplicated in addition to two initial molecules which allow duplication steps. Initial molecules are those with short chains and four different chemical compositions or Nucleotides which form various branches of genetic material. Equipments needed for PCR include reaction tube, chemical reagents, and heat source (Shah-Hoseini et al., 2006a; Shah-Hoseini et al., 2006b).

2. Materials and Methods

2.1 Sample collection

Drinking water samples were collected from 18 drinking water wells in different villages of Qom province for test. Water samples were collected in 250 ml glass containers with a polypropylene autoclavable lid for PCR and MPN test.

2.2 MPN test

MPN test was carried out according to method provided in method standard book in 10-tube form. In first stage of MPN test, 10 ml of sample water was added to tubes containing 10 ml Lauryl Triptose broth by Durham tube. Test tubes were heated for 48 h at 37 °C and were examined in terms of acid and gas production. Then, a complete loop of every positive tube in probability stage was transferred to test tubes containing Brilliant Green Lactose Bile broth by Durham tube in confirmatory stage. Test tubes were heated for 48 h at 37 °C. Gas formation in Durham tubes indicated positive response in this stage. MPN was calculated for positive response tubes in this stage of test using MPN standard table.

2.3 DNA extraction

The first step in PCR test is separating bacteria's genetic material or DNA. To this end, 100 ml of test water sample was centrifuged using falcon tubes. The supernatant was discarded and 100 microliter of water containing one-tenth percent of autoclaved diethylpyrocarbonate was added to tube. Then, tube was vortexed severely so that cells attached to tube wall are separated. This liquid was then transferred into a 1.5 ml microtube using sampler. Obtained solution was centrifuged for 10 min at 12,000 rpm, and the supernatant was discarded. 467 microliter of TE buffer (10 mM Tris, 1mM EDTA, pH 8.0), 30 microliter of 10% SDS and 3 microliter of 20 mg/L Proteinase K were added for lysis of bacterial cells, and it was heated for 30 min at 37 °C. 500 microliter phenol was added to resulting lysate so that organic materials are

eliminated. Following mixing sample with phenol by vortex, it was centrifuged for 10 min at 12,000 rpm. Top aqueous phase was transferred into a new microtube after centrifuge. In order to remove a small amount of phenol present in aqueous phase which could act as inhibitor in PCR test, chloroform was added as the same volume of aqueous phase, and again it was centrifuged for 5 min at 12,000 rpm. Resulting aqueous phase was carefully transferred to other tube. In order to precipitate and concentrate DNA, 3 M sodium acetate solution was added in volume equal to 1/10 of sample volume. After it was mixed, absolute ethanol was added in volume equal to twice the sample volume and it was put in - 20 °C temperature for 20 min. Following incubation time expiration at the cold, sample was centrifuged for 20 min at 12,000 rpm so that insoluble DNA was precipitated. Then precipitated DNA was rinsed by 200 microliter ethanol alcohol 70%, and alcohol was removed following centrifuge for 5 min. subsequently, 100 microliter of TE buffer was added to precipitation and sample was transferred to PCR test stage. In addition, spectrophotometery in wavelengths of 260 and 280 nm was used before transferring samples to duplication stage so that extracted nucleic acids were evaluated.

2.4 Polymerase chain reaction

In order to identify Coliforms, Lac Z genomic segment was used, which encodes β-galactosidase enzyme in Colifrom bacteria. Thus, specific *forward and reverse primers of this genomic segment were used as follows:*

Forward: 5'ATGAAAGCTGGCTACAGGAAGGCC3' (from 392 to 415)

Reverse: 5'GGTTTATGCAGCAACGAGACGTCA3' (from 633 to 656)

All reagents used in polymerase chain reaction were obtained from CinnaGen Co., and the primers were designed by Nedaye Fan Co. Concentration of PCR elements were as follows: 2.5 mM magnesium ion, 0.5 mM of each primer, 200 mM of each deoxynucleoside triphosphate, 2 unit of Taq DNA Polymerase enzyme, 25 microliter of PCR buffer in 1X concentration, then final volume was reached to 100 microliter by sterile distilled water. Genomic duplication plan was performed using thermal cycler machine for 30 thermal cycles as follows: initial denaturation at 95 °C for 5 min, subsequent denaturations at 94 °C for 1 min, connecting primers to pattern DNA at 55 °C for 1 min, duplication of target gene at 72 °C for 1 min, and final duplication at 72 °C for 2 min. Agar gel 2.5% in TBE buffer was used for final evaluation of PCR product. In addition, DNA of E.coli and autoclaved deionized water were used as positive and negative controls, respectively. Samples were stained with ethidium bromide before electrophoresis, and following electrophoresis they were investigated under UV using transilluminator device.

3. Results

3.1 MPN test results

MPN test results indicated 3 wells among a total of 18 drinking water wells as negative in confirmatory stage (Table 1).

3.2 PCR test results

In PCR test it was found that test result was positive regarding presence of Lac Z gene in 8 wells out of total of 18 drinking water wells. PCR test results are shown in Table 2.

The band related to 264 bp genomic segment on agar gel was clearly visible under UV in electrophoresis of PCR products in positive samples. In addition, similar band was also seen for DNA of E-coli used as positive control. On the other hand, no band was seen along with the well related to Sterile distilled water used as negative control.

4. Discussion and Conclusion

Some problems are raised regarding conventional culturing methods for routine bacteriologic monitoring of water supply resources including presence of VBNC bacteria, low sensitivity, accuracy and speed in conventional monitoring methods as well as lack of an exclusive feature for identification of real fecal Coliforms (E.coli) in common laboratory methods. Such problems make it necessary to seek for novel methods without mentioned drawbacks (Bej et al., 1991; Byrd et al., 1991; Shahian et al., 2009). PCR technique which detects molecular genetic material of organisms in water has unique characteristics distinguishing it from other conventional laboratory techniques. Additionally, importance of VBNC cells in

human infection has been identified and it seems that cells remain pathogenic in this condition (Shahian et al., 2009). Regarding the fact that bacteria cannot be detected by conventional methods in this condition, contaminated drinking water can be a threat to public health. In order to avoid such a situation it seems necessary to use new methods for detecting microorganisms in drinking water quality control laboratories, especially in critical situations where high speed and accuracy tests are very important and vital (Rompré et al., 2002). It should be noted that using novel techniques of water microbial monitoring does not mean completely elimination of conventional methods, rather using such techniques in parallel and together helps experts in obtaining appropriate and more practical solutions.

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Table 1. MPN test results

Well No. / Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
MPN/100ml					3.6				5.1			3.6						

Table 2. PCR test results

Well No. / Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
PCR		+		+	+		+		+			+	+			+		

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