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Comparison of Multiple Tube Fermentation and Polymerase Chain Reaction Methods for the Detection of Coliforms in Freshwater

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Abstract

Background and objective: Detection of microbial pathogens in water is one of the major health issues. *Escherichia coli* species are used as indicators of fecal contamination in water microbial detection. In this study, efficacies of two methods of multiple tube fermentation and polymerase chain reaction have been compared for the detection of coliforms (especially *Escherichia coli*) in water.

Material and methods: To compare multiple tube fermentation and polymerase chain reaction methods, 15 water samples were collected from five different sources (three gutter, six well, three tap and three bottled mineral water samples). The samples were cultured in lactose broth media to achieve the most probable number of bacteria. Furthermore, acetate cellulose filter method was used for the bacterial DNA extraction to investigate *lac*Z (indicating the presence of coliforms) and *uid*A (indicating the presence of *Escherichia coli*) genes.

Results and conclusion: Based on the results of multiple tube fermentation, eight (53.3%) and six (40%) samples were contaminated with coliforms and *Escherichia coli*, respectively. Furthermore, polymerase chain reaction results showed that ten (66.7%) and eight (53.3%) samples contained coliforms and *Escherichia coli*, respectively. Results have suggested that polymerase chain reaction is much faster, more accurate and more sensitive than traditional methods (e.g. multiple tube fermentation) for the detection of coliform contaminated water. Moreover, several types of bacteria can be tracked simultaneously by M-PCR.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Water-borne infections threaten the public health seriously [1-3]. Clean drinking water is still a critical issue for the governments [4,5]. Water contaminated with pathogenic microorganisms causes infectious diseases and its outcomes such as severe illness and death in a large scale within a short time [6]. The most commonly found pathogens in water are intestinal pathogens [7]. Since most cases of water pollution are associated with animal and human wastes, coliform bacteria have been chosen as contamination indicators [8-10]. Conventional methods such as multiple tube fermentation (MTF), for the identification of bacterial contamination, have many disadvantages such as lack of accuracy, high rate of false results, time consuming and inability to detect the bacteria that are not cultivable. Therefore, novel methods for the identification of pathogens in water such as polymerase chain reaction (PCR) have been developed. These methods are further sensitive and accurate, much faster and capable of detecting uncultivable and fastidious bacteria in food and water [11-13]. Moreover, they cost less for routine multiple-sample testing as they use small amounts of reagents. Indeed, the conventional test costs have been risen significantly in recent years. In general, drinking water must be pathogen free due to the public health

concern. Since coliforms (e.g. *Escherichia* (*E.*) *coli*) are common contaminants of the water sources, detection of these bacteria is important. This requires further development and optimization of rapid and accurate detection methods to guarantee the safety of drinking water.

Since comparison of these methods had not previously been reported in Tehran or for the genes or water samples used, the current study was carried out to compare MTF and PCR methods for the qualification of freshwater samples in Tehran, Iran, to determine if molecular methods could efficiently replace traditional methods as novel promising methods.

2. Materials and methods

In this study, 15 water samples with various sources were examined. These included three gutter, six well, three tap and three bottled mineral water samples collected in Tehran, Iran, 2012-13, according to the Standard Sampling Protocol No. 2347, published by the Institute of Standards and Industrial Research of Iran (ISIRI).

Multiple tube fermentation (MTF)

Samples were cultured rapidly in lactose broth (LB) containing Durham tubes (9-tube fermentation method) to reach the most probable number of bacteria (MPN). Tubes were incubated at 35.5°C for 24-48 h and results were recorded. Then, 50 ml of each positive sample were cultured into two tubes containing brilliant green agar (BGLB) and incubated at 35°C for 48 h and 44°C for 24 h, respectively. Then, 50 ml of the positive sample (incubated at 44°C) were cultured in *E. coli* broth and incubated at 44°C for 24 h. Results from LB, BGLB (35°C) and BGLB (44°C) cultures indicated the total count of bacteria and presence of coliforms and fecal coliforms, respectively. Results from the EC broth showed the presence of *E. coli* in samples.

Polymerase chain reaction (PCR)

In general, 1 l of each sample (250 ml for gutter water samples) was passed through 0.42-µm cellulose acetate filter papers. The bacterial DNA was extracted from the filters using DNA extraction kit (Bioneer, South Korea) and then used in PCR based on an original protocol by Mazaheri Nezhad Fard et al, 2011 [14]. The *lac*Z primer pair 5'-ATGAAAGCTGGCTACAGGAAGGCC-3' and 5'-GGT-TTATGCAGCAACGAGACGTCA-3', encoding 264-bp products, was used to detect total coliforms. The *uid*A primer pair 5'-AAAACGGCAAGAAAAAGCAG-3' and 5'-ACGCGTGGTTACAGTCTTGCG-3', encoding 147-bp products, was used to detect *E. coli* (Figures 1 and 2). Two amplicons, representing *lac*Z and *uid*A genes, were sequenced using Sanger method and data were compared with data annotated in genetic databases (EMBL Accession Nos. HE984350 and HE984351). An *E. coli* O157:H7 ATCC 35218 was used in PCR as positive control.

Multiplex polymerase chain reaction (M-PCR)

Conditions were the same as those of the single PCR, except that the concentration of primers reduced to 0.2 pM. Thermal cycling conditions included an initial denaturation at 95°C for 5 min followed by 30 cycles; each cycle included denaturation at 94°C for 1 min, annealing at 58-62°C (Gradient PCR) for 1 min and extension at 72°C for 1 min. Final extension was carried out at 72°C for 7 min (Figure 3).

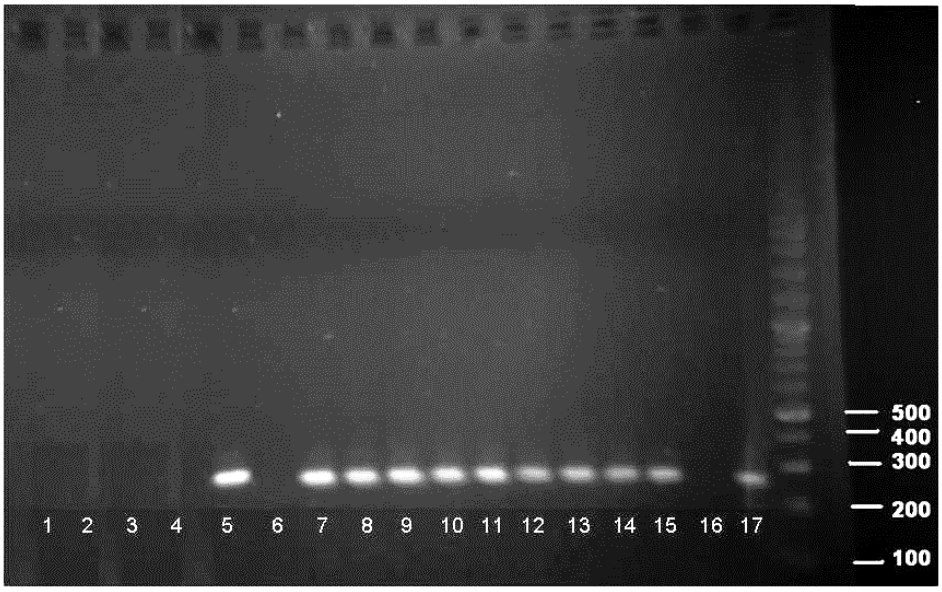

Figure 1. 264-base electrophoresed *lac*Z PCR products. Lanes 1, 2 and 3: mineral water; Lanes 4, 5 and 6: well water; Lanes 7, 8 and 9: gutter water; Lanes 10, 11 and 12: tap water; Lanes 13, 14 and 15: well water; Lane 16: negative control; Lane 17: positive control

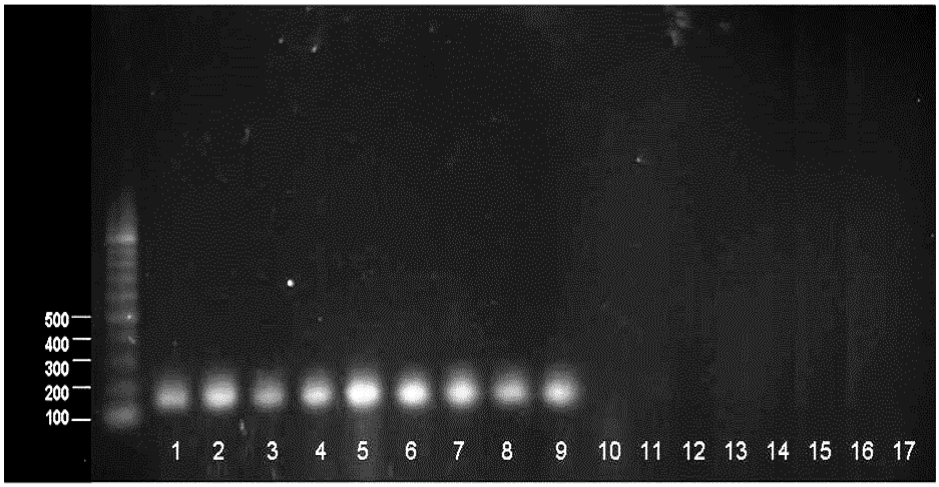

Figure 2. 147-base electrophoresed *uid*A PCR products. Lanes 1, 2 and 3: gutter water; Lane 4: positive control; Lanes 5, 6 and 7: well water; Lanes 8, 9 and 10: tap water; Lanes 11, 12 and 13: mineral water; Lanes 14, 15 and 16: well water; Lane 17: negative control

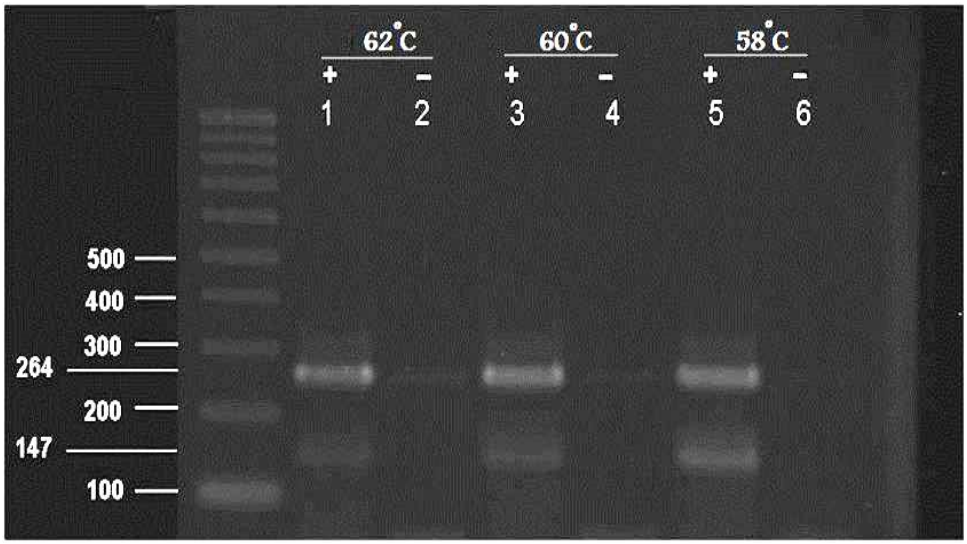

Figure 3. Electrophoresed gel of 147-base *uid*A and 264-base *lac*Z M-PCR products. Lanes 1 and 2: positive and negative controls at 62°C; Lanes 3 and 4: positive and negative controls at 60°C; Lanes 5 and 6: positive and negative controls at 58°C

Statistical analysis

Statistical analysis was carried out using SPSS v.18 Software (IBM Analytics, USA). Chi-square test and Fisher's exact test were used for the analysis. The $P \leq 0.05$ were reported as significant. All experiments were carried out in duplicate.

3. Results and discussion

Of 15 samples, eight samples (53.3%) included coliforms; from which, six samples (40%) were contaminated with fecal coliforms and *E. coli* ($P \leq 0.05$) (Table 1). The PCR results showed that ten samples (66.7%) included coliforms; from which, eight samples (53.3%) were contaminated with *E. coli* (Table 2). In this study, *lac*Z gene-specific primers were used for the detection of coliforms since conventional methods were based on the beta-galactosidase enzyme (the *lac*Z gene product) [15-17]. Furthermore, *uid*A gene was candidate for the detection of *E. coli*. This gene encodes beta-D-glucoronidase enzyme. In studies, *uid*A has been used to detect *E. coli* successfully [3,18]. Results of this study indicated that use of PCR included advantages such as time saving. Bej et al. showed that use of PCR, instead of traditional methods that worked using beta-D-glucoronidase (MUG) enzymes, gave better results [16]. Since many strains of *E. coli* (e.g. *E. coli* O157:H7) are MUG negative, PCR could be used for the detection of these strains, targeting *uid*A gene.

Table 1. Results for multiple tube fermentation method based on the most probable number table

Sample	Source	Total colony	Total coliform	Fecal coliform	*Escherichia coli*
1	Gutter water 1	> 1100	> 1100	> 1100	> 1100
2	Gutter water 2	> 1100	> 1100	> 1100	> 1100
3	Gutter water 3	> 1100	> 1100	> 1100	> 1100
4	Well water 1	> 1100	> 1100	240	93
5	Well water 2	240	240	93	43
6	Well water 3	460	460	93	43
7	Mineral water 1	3	-	-	-
8	Mineral water 2	< 3	-	-	-
9	Mineral water 3	< 3	-	-	-
10	Tap water 1	< 3	-	-	-
11	Tap water 2	4	4	-	-
12	Tap water 3	23	9	4	-
13	Well water 4	< 3	-	-	-
14	Well water 5	< 3	-	-	-
15	Well water 6	< 3	-	-	-

MPN= Most Probable Number

Table 2. PCR results of *lac*Z and *uid*A genes in water samples

Sample	Source	DNA conc.*	*lac*Z	*uid*A
1	Gutter water 1	99.6	+	+
2	Gutter water 2	94.56	+	+
3	Gutter water 3	105	+	+
4	Well water 1	43.44	+	+
5	Well water 2	36.96	+	+
6	Well water 3	39.6	+	+
7	Mineral water 1	0.72	-	-
8	Mineral water 2	0.6	-	-
9	Mineral water 3	1.68	-	-
10	Tap water 1	26.04	+	-
11	Tap water 2	39.48	+	+
12	Tap water 3	52.08	+	+
13	Well water 4	13.92	-	-
14	Well water 5	14.64	+	-
15	Well water 6	17.28	-	-

*mg ml^{-1}

In the current study, present of bacteria in various dilution samples was verified using culture methods as well as PCR. The PCR data showed that this method was able to detect bacteria in water filtrates more precisely than the culture method was. Studies have shown high sensitivity of PCR compared to MPN, even without DNA purification [19]. Therefore, the risk of false negative results decreases. Soltan Dallal et al. collected water samples from wells in parks in Tehran to evaluate the genetic diversity of *E. coli* strains using Multiplex PCR [5]. Results showed that of 165 samples, 90 samples were contaminated with *E. coli* and 67 with pathogenic strains which caused diarrhea. Alternatively, successful PCR amplifications were achieved by cells concentrated with hydrophobic filters for the detection of all coliform bacteria, while false negative results decreased considerably [20]. A further advanced PCR, quantitative PCR (qPCR), is the most common method for the bacterial count in water samples as this method is very accurate and is not affected by the bacteria physiological status, in contrast to microbial culture methods. Furthermore, this method is very fast and results can be obtained within 2-3 h due to the logarithmic increasing of desired sequences [21].

4. Conclusion

In general, PCR has been described as an accurate method for the detection of specific microorganisms. This method (proportional to RT-PCR) cannot reveal viability, vitality or the number of target microorganisms since the results are only based on the presence of microbial genes. Furthermore, water chemical pollution may result in inhibition of DNA polymerases. Modification and optimization of PCR-based methods are necessary for a better and successful setup. In summary, results from this study and other similar studies show that molecular methods such as PCR are more accurate and rapid (8-10 h in PCR instead of 72-96 h in MFT) to detect microbial contamination in water than traditional methods such as MTF are. Advantages and disadvantages of both methods are listed in Table 3.

5. Acknowledgements

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6. Conflict of interest

The authors declare no conflict of interest.

Table 3. Comparison of polymerase chain reaction and multiple tube fermentation methods: advantages and disadvantages

Method	Advantage	Disadvantage
PCR	• Faster (8-10 h) • More sensitive and accurate • Ability to detect several samples simultaneously • Ability to detect killed bacteria • Ability to detect damaged bacteria by chlorination • Ability to detect several types of bacteria (M-PCR) • Ability to count bacteria accurately (q-PCR)	• More expensive • Requires special equipments and skilled lab technicians • Requires careful setup before starting • Inability to differentiate live and dead bacteria
MTF	• Ability to estimate the number of bacteria • Ability to differentiate live and dead bacteria • No need for experts • Cheaper • Requires routine equipment	• Inability to detect damaged bacteria by chlorination • Inability to track different bacteria simultaneously • Inability to grow uncultivable bacteria • Less sensitive and accurate • Time consuming (72-96 h) • Risk of spreading infectious diseases • Requires space-consuming equipment and time-consuming preparations

PCR= polymerase chain reaction; MTF=multiple tube fermentation

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مقایسه دو روش تخمیر چند لوله‌ای و واکنش زنجیره‌ای پلیمراز برای ردیابی کلیفرم‌ها در آب شیرین

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چکیده

سابقه و هدف: شناسایی میکروب‌های بیماریزا یکی از مسایل مهم بهداشت آب به شمار می‌آید. گونه‌های *اشرشیا کلی* به عنوان نشانگر آلودگی مدفوعی در آب مورد استفاده قرار می‌گیرند. در این مطالعه، میزان کارایی دو روش تخمیر چند لوله‌ای و واکنش زنجیره‌ای پلیمراز در شناسایی کلیفرم‌ها (به ویژه *اشرشیا کلی*) در آب مورد مقایسه قرار گرفته اند.

مواد و روش ها: به منظور مقایسه روش‌های تخمیر چند لوله‌ای و واکنش زنجیره‌ای پلیمراز، ۱۵ نمونه آب از پنج منبع گوناگون (سه نمونه از آب رودخانه، شش نمونه از آب چاه، سه نمونه از آب شیر و سه نمونه از آب بطری) جمع‌آوری شد. نمونه‌ها بر محیط کشت لاکتوز براث به منظور تعیین بیشترین شمارش احتمالی باکتریایی کشت داده شدند. علاوه براین، برای بررسی ژن‌های *lacZ* (معرف حضور کلیفرم) و *uidA* (معرف حضور *اشرشیا کلی*) از روش فیلتر استات سلولز برای استخراج DNA باکتریایی استفاده شد.

یافته‌ها و نتیجه‌گیری: بر اساس نتایج به دست آمده از روش خمیر چند لوله‌ای، هشت (۵۳/۳٪) و شش (۴۰٪) نمونه‌ها به ترتیب به کلیفرم و *اشرشیا کلی* آلودگی بودند. علاوه بر این، نتایج روش واکنش زنجیره‌ای پلیمراز نشان داد که ده (۶۶/۷٪) و هشت (۵۳/۳٪) نمونه‌ها به ترتیب به کلیفرم و *اشرشیا کلی* آلوده بودند. نتایج نشان می‌دهد که برای تشخیص آلودگی آب به کلیفرم، روش واکنش زنجیره‌ای پلیمراز بسیار سریعتر، دقیق‌تر و حساس‌تر از روش سنتی (مانند روش خمیر چند لوله‌ای) می‌باشد. به علاوه، با روش واکنش چندگانه زنجیره‌ای پلیمراز (M-PCR) چندین نوع باکتری به طور همزمان قابل ردیابی می‌باشند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۱۹ آگوست ۲۰۱۸
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پذیرش ۲۴ نوامبر ۲۰۱۸

واژگان کلیدی

- کلیفرم‌ها
- *اشرشیا کلی*
- آب شیرین
- تخمیر چند لوله‌ای
- واکنش زنجیره‌ای پلیمراز

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