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Development and Validation of Multiplex PCR for the Simultaneous Detection of Total Coliform Bacteria, *Escherichia coli* and *Clostridium perfringens* in Water

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ABSTRACT

Background: Water is an important source of numerous infectious diseases in humans. The most important indicator bacteria include *Escherichia coli* and total coliform species. *Clostridium perfringens* is a general indicator of treatment efficiency.

Methods: Diagnosing microbial contamination of water by culture method, in addition to high cost, has low speed and many limitations. The PCR (Polymerase Chain Reaction) method is a promising alternative for simultaneous detection of these bacteria. In this research, we developed multiplex PCR for detection of *lacZ*, *uidA* and *plc* genes as an indicator of the presence of total coliforms, *Escherichia coli* and *Clostridium perfringens*. In the following, 33 samples of water and wastewater from Razavi Khorasan province were tested using standard culture methods and this multiplex PCR for comparison.

Results: Multiplex PCR was not only confirmed by cultural results but also has more accuracy and sharpness. The results of the validation tests showed that our multiplex PCR method had a significant advantage over the conventional culture method.

Conclusion: The developed multiplex PCR method demonstrated superior accuracy and efficiency compared to traditional culture techniques, offering a reliable alternative for rapid detection of waterborne pathogens.

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Introduction

Water contamination by pathogenic microorganisms is a serious threat to human health (1). Access to safe drinking water sources has become an important issue and even a problem in many countries of the world (2). The pollution of drinking water sources is of particular importance from the health point of view and requires serious attention (3). To carry out this research, the most important pollution index bacteria were used to evaluate the potential risk of drinking water for public health, including *Escherichia coli*, coliform types, and *Clostridium perfringens* as a general indicator of purification efficiency (4). Conventional cultural methods such as the presence or absence of the mentioned pollution indicators are key elements of most drinking water quality guidelines. The four genera, *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter*, are usually accepted as including the entire coliform population. Those coliforms that have the ability to ferment lactose at 44 ±0.5 °C are considered as a subset of total coliforms called thermotolerant coliforms (formerly known as fecal coliforms) (5). *Clostridium perfringens* has a longer lifespan in the environment and greater resistance to adverse environmental conditions and disinfectants, which makes it able to survive in soil and biofilm for years. For this reason, *Clostridium perfringens* should be used as a more suitable indicator for the presence or absence of organisms in water treatment units, as well as for the presence or absence of *Cryptosporidium parvum* oocysts (6-9).

Considering that the detection of microbial contamination of water by traditional culture-based methods such as PA (Presence-Absence) and Multiple Tube Fermentation (MTF) methods is expensive and has a low speed and especially cannot detect the viable but nonculturable) VBNC (bacteria. Therefore, we have developed a high efficiency multiplex PCR method for water bacteriological health assessment (10, 11). This method is a fast and reliable tool for evaluating the microbiological quality of water and it can be an alternative to conventional methods for the screening of water samples. In this research, the standard culture methods of culture were used to compare them with the multiplex PCR method designed for *lacZ* gene of total coliforms and *uidA* for fecal coliforms and Plc for *Clostridium perfringens* (12, 13).

Materials and Methods

Sample collection

The target population of this research is the water sources of Razavi Khorasan province. 33 samples of spring, aqueduct and water treatment package plant were randomly selected. They were transported to the water laboratory of Razavi Khorasan Province Water and Sewerage Company and were tested maximum six hours after sampling.

Detection of target bacteria

Standard culture methods and PA and MTF methods were used to detect total coliforms, *E. coli*, and membrane filter For *Clostridium perfringens* (5, 14).

Molecular detection

Primers

After ensuring the desired sequences, the selected primers were synthesized by Metabion – Germany. The Plc gene of *Clostridium perfringens* is 296bp in size, with forward and reverse oligo code 231013B061F061/6 and 231013B061-G062/6, respectively. The uidA gene of *E. coli* is 390bp in size, with forward and reverse oligo code 231013B061H063/6 and 231013B061A074/6, respectively. The *lacZ* gene of total coliform is 173bp in size, with forward and reverse oligo code

231013B061B075/6 and 231013B061C076/6, respectively.

After the design, the primers were received in lyophilized form. To prepare the storage solution, it was done according to the instructions (analysis sheet) included with the primers. The bioinformatics specificity of primers was evaluated and confirmed by Nucleotide BLAST.

Plc, *uidA*, *lacz* gene certification respectively; *Clostridium perfringens* plc Genome (Lot No: 3692501), *Escherichia coli uidA* Genome (Lot No: 3692502), total coliform *lacz* Genome ((Lot No: 3692503). Provided by Invitrogen by Thermo Fisher Scientific.

Polymerase chain reactions for molecular detection

Determination of the optimal temperature (temperature gradient) from 51 °C to 60 °C was evaluated. The time schedule given to the thermal cycler device to perform the PCR reaction was mentioned in Table 1.

Table 1. PCR Steps: Time schedule given to the thermal cycler device to perform the PCR .

Initial Denaturation	10 min	95 ºC
Denaturation	1 min	95 ºC
Annealing	1 min	51-60 °C
Extension	1 min	72 ºC
Final extension	10 min	72 ºC
35 cycles		

To set up the multiplex PCR by changing the concentration of primer and genome of total coliform was done. That is the case different concentrations of the primer were set to identify different concentrations of the template.

Time schedule given to the thermal cycler device to perform the PCR reaction for determining the optimal concentration was mentioned in Table 2.

The sensitivity of multiplex PCR was evaluated according to different concentration of genetic materials. To find the limit of detection (LOD), in fact, finding the lowest concentration of detectable genetic materials from the concentration of 10 picograms per microliter to 1 femtogram per microliter was evaluated.

Table 2. PCR Steps: Time schedule given to the thermal cycler device to perform the PCR reaction for determining the optimal concentration.

Initial Denaturation	10 min	95º C
Denaturation	1 min	95º C
Annealing	1 min	59 °C
Extension	1 min	72º C
Final extension	10 min	72º C
35 cycles		

In order to compare and evaluate the contamination of pathogens of *E. coli*, *Clostridium perfringens* and total coliform, 33 samples (spring, aqueduct and water treatment package plant) were collected from different fields of Khorasan using culture method and multiplex PCR (Table 3).

Results

The results of the temperature gradient (from 51 °C in well 2 to 60 °C in well 10) were evaluated (Table 4). According to the bands of wells 2-10 in electrophoresis gel, the sharpest band belongs to well number 9 (The temperature gradient mentioned in Table 4). Therefore, the temperature of 59 °C was chosen as the optimal temperature (Figure 1).

The electrophoresis image shows well the sharp band in the concentration changes applied in wells 2 and 4. In order to be economical, the concentrations applied in well number 2 were accepted (Figure 2).

The band of well number 10 is evident in the gel electrophoresis results of Figure 3 (DNA with a concentration of 1 femtogram). Therefore, the answer to the question of what concentration of

Table 3. Sample types: 33 samples (spring, aqueduct and water treatment package plant) were collected from different fields of Khorasan.

NO Sample	Types of samples		
	Spring	Aqueduct	Water treatment package plant
1		*	
2		*	
3		*	
4		*	
5		*	
6		*	
7		*	
8		*	
9		*	
10		*	
11		*	
12		*	
13	*		
14	*		
15	*		
16	*		
17			*
18			*
19			*
20			*
21			*
22			*
23			*
24			*
25			*
26			*
27			*
28			*
29			*
30			*
31			*
32			*
33			*

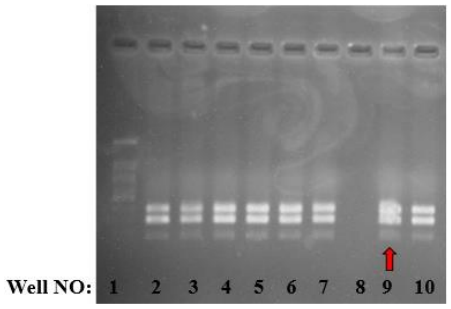

Fig 1. Results of gel electrophoresis with the aim of determining the temperature gradient. The first well belongs to the ladder. The lower band belongs to total coliform; the middle band belongs to *Clostridium perfringens* and the upper band belongs to *E. coli*. The sharpest bar belongs to well number 9, indicated by the red arrow.

Table 4. Temperature gradient: Determining the optimal temperature (temperature gradient).

Well Number	Temperature (°C)
1	Ladder 100bp
2	51
3	51/6
4	52/7
5	54/4
6	56/6
7	58/3
8	-
9	59
10	60

DNA the designed multiplex PCR can handle is equal to the concentration of 1 femtogram as the LOD. The serial of DNA concentration mention in Table 5.

Comparing and evaluating the contamination of three pathogens, *E. coli*, *Clostridium perfringens* and total coliform, 33 samples (spring, aqueduct and treatment plant) from different parts in Khorasan using the triplex culture method and multiplex PCR. The results showed that the

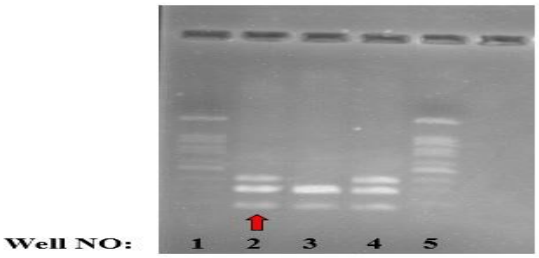

Fig 2. Gel electrophoresis of the PCR reactions optimization. The lower band: total coliform, the middle band: *Clostridium perfringens*, the upper band: *E. coli*. Well 2: primer concentration. Well 4: template concentration.

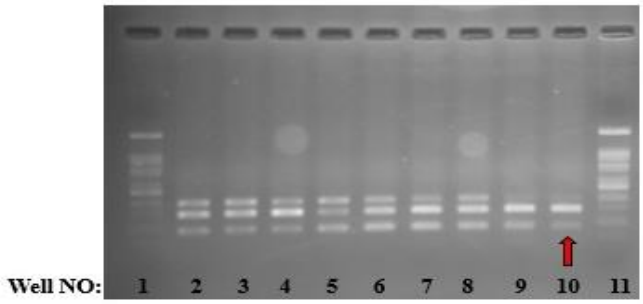

Fig 3. Gel electrophoresis of PCR optimization. The lower band: total coliform, the middle band: *Clostridium perfringens*, the upper band: *E. coli*. Well 10: the LOD (DNA with a concentration of 1 femtogram).

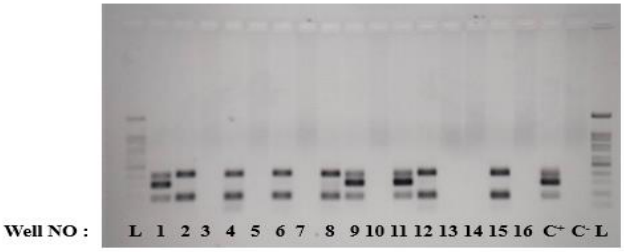

Fig 4. Gel electrophoresis results of 33 samples. A: From left to right: L(ladder), 1-16 samples, C+ (positive control), C- (negative control), L(ladder). B: From left to right: L(ladder), 17-33 samples, C- (negative control), C+ (positive control).

Table 5. DNA concentration: The serial of DNA concentration to evaluation of the sensitivity of the multiplex PCR.

Well Number	DNA Concentration
1	Ladder 100bp
2	10pg/ µl
3	5pg/ µl
4	1pg/ µl
5	0.5pg/ µl
6	100fg/ µl
7	50fg/ µl
8	10fg/ µl
9	5fg/ µl
10	1fg/ µl
11	Ladder 100bp

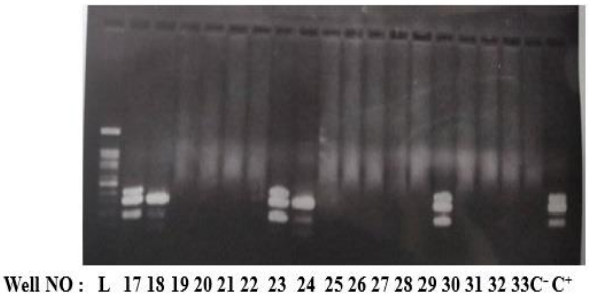

Fig 5. Gel electrophoresis results of 33 samples. A: From left to right: L(ladder), 1-16 samples, C+ (positive control), C- (negative control), L(ladder). B: From left to right: L(ladder), 17-33 samples, C- (negative control), C+ (positive control).

Table 6. Culture and PCR comparision: The results of comparing and evaluating the contamination of three *E. coli* pathogens, *Clostridium perfringens* and total coliform, from 33 samples (spring, aqueduct and treatment plant) from different parts of Khorasan using the triplex culture method and multiplex PCR.

NO Sample	Types of samples			Culture			PCR		
	Spring	Aqueduct	water treatment package plant	*Clostridium perfringens*	Total coliform	*E. coli*	*Clostridium perfringens*	Total coliform	*E. coli*
1		*			*	*	*	*	*
2		*			*	*		*	*
3		*							
4		*						*	*
5		*							
6		*			*			*	*
7		*							
8		*			*			*	*
9		*		*	*	*	*	*	*
10		*							
11		*			*	*	*	*	*
12		*			*	*		*	*
13	*								
14	*								
15	*				*			*	*
16	*								
17			*	*	*	*	*	*	*
18			*				*		
19			*						
20			*						
21			*						
22			*						
23			*	*	*	*	*	*	*
24			*				*		
25			*						
26			*						
27			*						
28			*						
29			*						
30			*	*	*	*	*	*	*
31			*						
32			*						
33			*						

detection method of C*lostridium*, and total coliform with the culture method was not able to detect 4, 4 and 1 samples, respectively, in contrast to the PCR method (Table 6).

Discussion

Limitations of cultivation and general identification methods include a lengthy incubation period, interference from other microorganisms, insufficient accuracy and sensitivity, and poor identification of VBNC bacteria (15, 16).

Molecular techniques have been suggested as a quick and reliable way to identify different forms rapidly. Some of these methods allow for the identification of specific bacteria, whether culturable or non-cultivable, in just a few hours, as opposed to traditional methods (17).

By utilizing multiplex PCR, multiple harmful organisms can be detected all at once in a single test, eliminating issues linked to traditional cultivation techniques (18).

By using multiplex PCR, it is possible to directly detect several pathogenic agents simultaneously in one test and avoid the problems associated with conventional culture methods (19).

Using multiplex PCR for *lacZ* total coliforms and *uidA* for *E. coli* and *plc* for *Clostridium perfringens* could be a rapid and dependable way to assess the bacterial quality of water, serving as a viable substitute for traditional culturing techniques (12).

Also, due to the ability of these bacteria to enter the VBNC state under stress, salinity, unfavorable environmental conditions or during applied processes such as disinfection or processes used in the food industry, the failure to identify these state Bacteria can cause serious risks. Compared to many pathogenic organisms such as *E. coli*, *Clostridium perfringens* has a longer lifespan in the environment and greater resistance to adverse environmental conditions and disinfectants, which makes it able to survive in soil and biofilm for years. For this reason, it has been suggested to use *Clostridium perfringens* as a more appropriate indicator for the presence or absence of viruses and protozoan cysts in water treatment units, as well as for the presence or absence of *Cryptosporidium parvum* oocysts. It should be used after disinfection with a mixture of oxidants (20).

According to studies, this is the first time that the multiplex PCR technique has been designed to simultaneously detect thermotolerant coliform bacteria (*E. coli*), total coliform bacteria, and *Clostridium perfringens* in water, and it has not been patented worldwide. This invention can be used to more quickly and accurately identify water pollution factors, especially coliforms, and as a result, it can significantly prevent the spread of this type of infection. So far, extensive studies have been conducted on the identification of water pollution factors, however, a limited number of them have used the multiplex PCR technique to detect these factors. In a study by ASIM K. BEJ and colleagues in 1991 in the United States, they used multiplex PCR to detect the target genes *lacZ* and uidA for the identification of total coliform bacteria and *E. coli*, respectively, for determining water quality, which had significant sensitivity (21).

In a study by Suwalee Tantawiwat and colleagues in 2005 in Thailand, the multiplex PCR technique of *lacZ*, *uidA*, and *plc* genes was used to simultaneously detect total coliform bacteria for *Escherichia coli* and *Clostridium perfringens* in drinking water, which was shown to be an effective, sensitive, and rapid method for simultaneously detecting these three microbiological parameters in drinking water (12).

In a study conducted by Si Hong Park and colleagues in 2011 in the United States, the aim was to develop a multiplex PCR that could identify and determine the three pathogens *Campylobacter*, *E. coli* O157:H7, and *Salmonella* in samples, which was able to measure and detect all three pathogens simultaneously in a single reaction (12).

In a study by Dehghan Fatemeh et al. in 2014 in Iran, rapid detection of coliforms was performed by amplification of *lacZ* and *uidA* genes in

multiplex PCR reaction compared to the probabilistic numerical method (MPN). This technique with short processing time and high sensitivity was used for simultaneous detection of total coliforms and *E. coli* in the distribution system of Arak city, which can be used as a primary screening test (22).

In a 2015 study by Felipe Molina and colleagues in Spain, two strategies were used to design oligonucleotide primers for the *LacZ* and *yaiO* genes for the simultaneous detection of total coliforms and *E. coli* by Multiplex PCR, which showed high specificity in identifying these agents (11).

In a study by G. Pandove in 2013, a multiplex PCR water test kit was designed that was able to simultaneously detect *E. coli*, *Y. enterocolitica*, and *A. hydrophila* in water (23).

In a 2016 study by Roohollah Kheiri et al. in Iran, two multiplex PCR methods were performed to simultaneously detect six waterborne bacteria including: *uidA* (*E. coli*), int (*Shigella* spp.), and *gyrB* (*Pseudomonas aeruginosa*), *invA* (*Salmonella* spp.), *ompW* (*Vibrio cholera*), and *lacZ* (coliforms) genes. The results showed that all primer pairs were specific only for their respective target organisms, and the detection sensitivity of both multiplex methods was high (10).

In a study by MA El-Leithy et al. in 2012 in Egypt, multiplex PCR was designed for the genes *stx1* (Shiga toxin 1 gene), *stx2* (Shiga toxin 2 gene), *eae* (intinimin gene), *hlyA* (hemolysin gene), *rfbE* (O157 antigen gene), and *fliC* (flagellar antigen gene) that had acceptable sensitivity (24).

In a study by El-Sayed Ahmed Kassem A. in 2015 in Egypt, a Multiplex PCR method was designed to detect the *lacZ* gene present in all coliform bacteria including *E. coli*, the *uidA* gene specific to *E. coli*, and the *tof* gene specific to all enterococci. This technique demonstrated an effective, sensitive, and rapid method for the simultaneous detection of these three microbiological indicators in contaminated water. This Multiplex PCR was used as an optimal method for the identification of contaminating microorganisms and the evaluation of three water treatment plants (12).

In a 2023 study by Arsyam Mawardi in Indonesia, multiplex PCR was used to detect the *lt-eae-stx2* target genes on ETEC-EPEC and EHEC, respectively. The method showed very good results, so that these findings can be considered as a reference for water analysis in several drinking water sources in Papua Province (12).

In a 2024 study in Poland by Bogumił Zimoń et al., multiplex polymerase chain reaction (PCR), using amplification of three genes (*cydA*, *lacY*, and *ydiV*), was used as a method to identify *E. coli* strains. This method had the advantages of rapid, inexpensive, and reliable detection of *E. coli*. The sensitivity and specificity of this technique were 95.76% and 99.49%, respectively. Due to the detection of three genes, this method is very cost-effective (25).

This study utilized the 3 genes using three primer pairs for simultaneous detection of 3 bacteria through water with a multiplex PCR protocol. According to the study findings, the multiplex PCR demonstrated the ability to identify bacterial targets even at a very low concentration of 1 femtogram of DNA, an achievement that is truly remarkable. The speed at which pollution is identified is the key factor in the water pollution crisis. The aim of this study was to develop a triple method to accurately and sensitively evaluate water pollution in a short amount of time. This triplex can rapidly identify three pathogens in about 4 hours; a major upgrade from traditional culture methods takes 4 days to 1 week. This method can reduce the time needed to identify bacteria, thus allowing for the quick detection of potential threats.

Conclusion

The purpose of this research was to create a triple combination to assess water pollution quickly, with great precision and sensitivity. This triplex is able to detect three pathogens quickly (around 4 hours), a significant improvement from conventional culture techniques (taking 4 days to 1 week). This approach can shorten the time needed to identify bacteria, making it suitable for detecting potential risks.

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Ethics approval and consent to participate

Not applicable.

Conflict of interest

The authors state that they have no conflicts of interest.

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