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# Determining Contamination by *Escherichia coli* in Water Resources through yaiO Gene Molecular Analysis, Iran

**Samaneh Ahmadi[1], Fatemeh Keshavarzi[2]***

*[1]Department of Biochemistry, Sanandaj branch, Islamic Azad University, Sanandaj, Iran*
*[2]Department of Genetics, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran*

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## ABSTRACT

*Recent studies have shown that Escherichia coli (E. coli) are the best indicator for evaluating fecal contamination and possible presence of enter pathogens. The method used at present in Iranian water laboratories for detection of these bacteria (the most probable number or the MPN) method. However, the use of the enzymatic method has expanded to overcome the limitations of the MPN method including its being very time-consuming (it requires 24-96 hours).Fifty drinking water samples were taken from drinking water resources in the rural areas of Kamyaran, the bacteria in the samples were isolated using the MPN test, and initial identification of the purified bacteria was carried out by employing a series of biochemical tests. The Oligo software was employed to determine the sequence characteristics of the designed primers, and the accuracy of the software was confirmed by using the BLAST software. Finally, the stages of genomic purification were carried out by employing the CTAB-NaCl method on the bacterial precipitation.Of the 50 samples taken from drinking ware resources of the rural areas in Kamyaran, 12 were positive in MPN tests. Moreover, use of the MPN test indicated that six of these 12 samples were infected with Escherichia coli. Presence of E. coliin all of the contaminated samples was confirmed using the PCR method and employing the yaiO gene-specific primers. Results showed that although the PCR method is a fast, reliable, and suitable method for detecting bacteria in drinking water, yet it is not possible to use it in the rural areas of Kamyaran at present due to shortages of facilities, laboratory materials and equipment, and specialist staff.*

**Key words:** *Escherichia coli*, Most Probable Number**,** Genomic Purification, PCR, yaiO Gene

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**Corresponding author**: Fatemeh Keshavarzi
**e-mail**✉ fkeshavarzi@iausdj.ac.ir
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## INTRODUCTION

Human life depends on safe water more than on anything else, and provision of safe water is one of the main goals in the development of any society[1].Contamination of drinking water is one of the ways through which various types of pathogens can be transmitted [2]. The World Health Organization (the WHO) recommends that the microbial quality of drinking water should be measured by using indicator bacteria, preferably *Escherichia coli* (*E. coli*). These bacteria have been selected to indicate fecal contamination instead of directly detecting the presence of pathogenic agents, with coliform bacteria being widely used as an indicator of water quality [3]. E. coliare the only member of the coliform group of bacteria that have been detected in mammalian intestines including those of humans, and their presence in water represents fecal contamination and may indicate the possible presence of pathogenic bacteria [4]. *E. coli* and fecal coliforms are very suitable for rapid detection of microbial contamination in drinking water [5]. Calcium hypochlorite powder is used for disinfection of drinking water in Iran, the optimum concentration of which, for contact time of 30 minutes and considering the pH value, ranges from 0.5 to 0.8 mg/l and reaches 1mg/l under epidemic conditions [1]. However, *E. coli* can hide among suspended particles and viruses and, hence, are not considerably affected by disinfection materials. Use of coliforms as indicator of water contamination dates back to the twentieth century

when MacConkey described the presence of lactose fermentative microorganisms[6].The usual methods of identifying coliforms in drinking water are very time-consuming and costly[7]. The polymerase chain reaction (PCR) technique is the most popular method in molecular biology for enzymatic replication of DNA without using a living organism, and allows multiple amplification of a small quantity of DNA molecules [8]. The PCR technique, developed by the American biochemist Karry Mullis, made it possible for researchers to amplify a small sample of DNA into millions of copies within a few hours [9]. The advantages of using the PCR method are its high speed, ability to identify bacteria that are in very small numbers, simultaneous detection of numerous bacteria, and very high sensitivity [10]. This research intended to identify *E. coli* in drinking water resources of the villages in Kamyaran using amplification of the yaiO gene.

## MATERIALS AND METHODS

### Sampling

Samples were taken from 18 wells, 22 common spring of Kamyaran villages. Samples were collected for bacteriological analysis in 300 ml capacity autoclaved glass containers, at 9 to 12 AM. The water samples were immediately taken up for microbial analysis to the water laboratory of health center in Kamyaran city. Most probable number tests (MPN) were used in for Bacteria isolation [9, 10]. MPN was conducted in three stage contains presumptive, confirmatory and completed test**.** Bacteria that have grown in completed test by Commercial phenotypic tests (API 20E) were identified [11].

### Primers

A bacterial colony was inoculated in culture LB broth for the purpose of genome extraction, and it was stored for one night in an incubator. When the bacterium showed logarithmic growth, the medium was divided into 1.5-ml tubes, which were centrifuged for 5 minutes at 5000 rpm and a temperature of $4\,^{\circ}C$. Afterwards, the upper solution was removed and the rest of the genome purification process was carried out on the bacterial sediment using the CTAB-NaCl method [12]. To isolate and multiply the yaiO gene from the bacterium, the PCR method was used. The PCR reaction was carried out with 2 primer pairs and Taqpolymerase enzyme. The product was exposed to electrophoresis on 1% agarose gel. The species for the PCR reaction were prepared with theTaq polymerase enzyme at a volume of 50 $\mu l$ as mentioned in Table 1.

**Table 1: PCR reaction with Taq polymerase enzyme**

| Substance | Volume ($\mu l$) | Final Concentration |
|---|---|---|
| Distilled water | 38 | -- |
| Magnesium Chloride (50 MM) | 3 | 3 MM |
| 10 X buffer | 5 | -- |
| Forward primer (20 Pmol) | 1 | 0.4 Pmol |
| Reversed primer (20 Pmol) | 1 | 0.4 Pmol |
| dNTPs (10 MM) | 1 | 0.2 MM |
| DNA pattern (genome) | 2 | 50 Ng |
| Taq DNA polymerase (5 U/μl) | 0.5 | 2.5 U |

The PCR program for the yaiO gene in the thermocycler for the Taq polymerase enzyme was arranged as Table 2.

**Table 2: The PCR program for the yaiO gene to be reacted with Taq polymerase enzyme**

| Stage | Temperature ($^{\circ}C$) | Time | Cycle |
|---|---|---|---|
| Initial denaturation | 94 | 5 min | 1 |
| Denaturation AnnealingExtension | 94 | 45 sec | 30 |
| | 57 | 30 sec | |
| | 72 | 45 sec | |
| Final extension | 72 | 7 min | 1 |

To isolate and multiply the yaiO gene from the bacterium, the PCR reaction was used. The PCR reaction was carried out with 2 primer pairs and Taq polymerase enzymes. The product was exposed to electrophoresis on 1% agarose gel [13]. The species for the PCR reaction were prepared with the Taq polymerase enzyme at a volume of 50 $\mu l$ as mentioned. In a reaction, genome of *E.Coli* bacterium was used as positive control.

### Assessment of Extracted DNA on Agarose Gel

To assess the quality of the extracted species, the agarose gel was used. In this method, the species were exposed to a 6 X loading buffer (1 $\mu l$ of loading buffer per 5 macroliter of the species) (Table 2) and were put in 0.7% agarose gel wells (0.7 gram of agarose powder was solved in 100 ml of TBE buffer and was boiled). Afterwards, using an electrophoresis tank and electrical current (1-5 V per 1 cm of gel) the species moved along the gel.

To observe the species on agarose gel, they were stained using ethidium bromide (with a concentration of 0.5 $\mu$g/ml), and quality of species was examined using a Gel document device (BioRad Gel Doc 1000) and UV radiation [13].

### Assessment of PCR Products Proliferated on Agarose Gel

To assess the quality of the extracted species, the agarose gel was used. The species were exposed to a 6 X loading buffer (1 $\mu l$ of loading buffer per 5 macroliter of the species) (Table 3) and were put in 1% agarose gel wells (1 gram of agarose powder was solved in 100 ml of TBE buffer and was boiled). Afterwards, using an electrophoresis tank and electrical current (1-5 V per 1 cm of gel) the species moved along the gel. To observe the species on agarose gel, they were stained using ethidium bromide (with a concentration of 0.5 $\mu$ g/ml), and quality of species was examined using a Gel document device (BioRad Gel Doc 1000) and with UV radiation.

**Table 3: Sequence of primers selected for gene proliferation**

| Primer | Nucleotides containing | PCR-product Size |
|---|---|---|
| yaiO Forward | 5′ TGATTTCCGTGCGTCTGAATG 3′ | 115 |
| yaiO Reverse | 5′ ATGCTGCCGTAGCGTGTTTC 3′ | |

## RESULTS

### A result of the biochemical tests

Table 4 presents results of identifying *E. coli* in biochemical tests. Of all the samples from which bacteria were isolated in the MPN test, six were contaminated with Escherichia coli, which were facultative anaerobic bacteria and grew with a metallic sheen on EBM agar.

### Results of identifying the isolated bacteria by using the PCR method

**Table 4: The results of identification of bacteria using biochemical tests**

| Tests | | | | | | | | | | | **Bacteria name** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $NO_2$ | *Methyl red* | Motility | Catalase | *Voges Proskauer* | $H_2S$ | *Simmons Citrate* | *Oxidase* | Beta-D-galactosidase | *Indole* | *Urea* | |
| + | + | + | + | - | - | - | - | + | + | - | *E. coli* |

Features of the reverse primer were determined using the Oligo software. Annealing of the primer required the temperature of 64.6˚C and the delta G (ΔG) of the primer for annealing to the target gene was -40.3cal/mole. Moreover, the primer had an internal loop with delta G (ΔG) of -3kcal/mole, and the maximum ΔG for primer-dimer formation was -4.7kcal/mole.

After the bacteria were cultured on LB, the bacterial suspension was exposed to isolation of chromosomal DNA, and PCR of the extracted genome belonging to the mentioned bacteria was carried out using the specific primers. In the genomic extraction from the bacteria, it was found that the DNA in the purified bacterial genome was not broken and enjoyed suitable quality and purity. Observation of 115-bp bands confirmed the presence of *E. coli* (Figure 1).

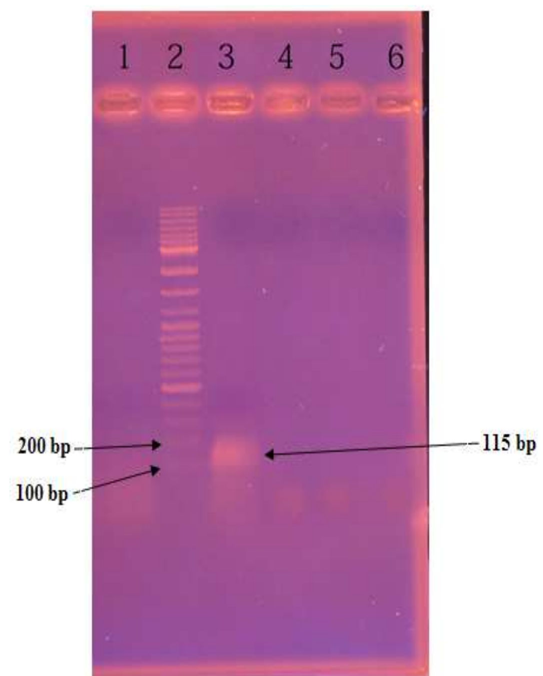


**Figure 1: Identification of E. coli by using primers specific for the yaiO gene(Column 1) PCR amplified products of the yaiO gene taken from bacteria number 1**

## DISCUSSION

According to the standards specified by the WHO, and as indicated in the Iranian standards, for drinking water, *E. coli* should not be detected in 100-milliliter samples of drinking water [1]. Development of primers for detection and identification of coliforms in drinking water is very difficult because coliform bacteria include a large group of many types of bacteria and, hence, the primers must be sufficiently specific. Bej et al. utilized the LacZ gene for rapid detection of coliform bacteria [14] and, in 1991, suggested the use of the uidA gene for rapid detection of these bacteria [15]. Fricker and Fricker 1994 successfully used the uidA gene that encodes the enzyme b-D glucuronidase (GUD)[16]. Pal P suggested utilization of the WecG, LacZ, and 16 rRNA genes for detection of coliform bacteria [4]. In research by K. Horkova, the primer sets suitable for obtaining complete coding sequences of LacZ and uidA genes were used to detect all coliforms and *E. coli*, respectively [17]. Results obtained by these researchers indicated that the PCR technique increased reliability of *E. coli* detection and prevented false positive results in biochemical tests. However, yaiO gene is recently used for rapid detection of E. coli. The most prominent feature of coliforms is that they contain LacZ gene that encodes the enzyme β-galactosidase, while amplification of yaiO gene is specifically used for detection of *E. coli* [18]. Nowadays, molecular methods for detection and identification of pathogens have attracted the interest of the international scientific communities because of their great speed and accuracy. The present research, which was conducted in the summer of 2015, intended to detect *E. coli* by using amplification of yaiO gene. Six of the 50 samples taken from the drinking water resources of the rural regions in Kamyaran were contaminated with *E. coli*. The PCR technique for detection of *E. coli* has been used before by Iranian researchers at laboratory scale [10]. However, this technique has never been adopted at a practical method due to shortages of laboratory facilities and of specialist staff. In the research by Hossein Aghababaee, a series of MPN tests was performed on 18 samples of drinking water taken from rural areas in Ghom, and it was noticed that three samples were positive[10]; however, the number of infected samples rose to eight when the PCR technique was employed. Sequences of the designed primers were studied with respect to annealing temperature, pairing of primers, and presence of loop primers by utilizing the Oligo software. After designing the primers, their accuracy was investigated by using the BLAST software, and it was found yaiO gene, taken from *E. coli* and present in the gene bank, exhibited the highest specificity.

## CONCLUSION

Results indicated 32 percent of the 50 samples taken from the drinking water resources of the rural regions in Kamyaran were contaminated with fecal coliforms, and 12 percent were infected with *E. coli*. Using Oligo and DNASIS, the primer suitable for proliferation of the gene fragment was designed, and the genome purification process was conducted on bacterial sediments using CTAB-NaCl. The primers designed for yaiO gene confirmed the presence of *E. coli* in all contaminated samples in all the contaminated samples. Although the PCR is a reliable and suitable technique for detecting bacteria in drinking water, yet it cannot be utilized in rural regions of Kamyaran due to shortages of facilities, laboratory materials and equipment, and specialist staff.

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