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Characterization of *Escherichia coli* Isolated from the Water Supplies in Meshkin Shahr, Iran

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Abstract

Water is the most important potable material with the highest consumption rate. Therefore, because of the growing population, providing healthy water always has been one of the major concerns for most countries. Water contamination tests are carried out based on indicator microorganisms. *E. coli* is the most important indicator for the detection of water contamination. *E. coli* classified into four main phylogenetic groups A, B1, B2, and D, which A and B1 are almost commensals and rarely can cause diseases in immune deficiency people, and groups D and B2 are cause extra-intestinal diseases.

This study aimed at the phylogenetic study of *Escherichia coli* in water supplies of Meshkin Shahr, Iran. 60 samples were collected and studied from two different water supplies. After cultivation and isolation, fecal coliforms were detected via the MPN method. *E. coli* isolation was confirmed by biochemical tests. The confirmed samples were phylogenetically investigated through multiplex PCR. Also, antimicrobial susceptibility was performed by using the Kirby-Bauer method for 14 antibiotics. The results showed that the average total count of coliforms was 620.467 cfu in 100 ml of water for Khiav Chayi and 92.750 cfu in 100 ml of water for Nahr Khatoon. The most prevalent phylogenetic groups were A (21.8%) and B1 (53.1%). 61% of the *E. coli* isolates were resistant to at least one antibiotic. Resistance to ampicillin was the most common among other antibiotics. The microbial load of Nahr Khatoon was lower than Khiv Chayi, so it is preferred to be used as a healthy water supply.

Keywords: Water; *E. coli*; Phylogenetic; Resistance; Antibiotic; MPN

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Introduction

Currently, it is a critical challenge to get access to enough and high-quality water. Climatic alterations and environmental pollutants have aggravated this problem. Despite extensive progress towards the Millennium Development Goals during recent years, organizations such as United Nations have reported that at least 11% of the world's population has no access to safe drinking water [1]. *Escherichia coli* is a Gram-negative rod-shaped bacterium belonging to order Enterobacteriales and family Enterobacteriaceae. This Bactria is the most important agent causing human urinary and gastrointestinal infections. Also, *E. coli* is the most valuable contamination index of water, food and agricultural products [2].

E. coli strains can be divided into one of the main phylogenetic groups: A, B1, B2, or D [3]. Strains of each phylogenetic category have different phenotypic and genotypic features. Furthermore, it has been reported that the four major phylogenetic groups differ in their ecological features. Phylogenetic group A and B1 are commensal and strains which are implicated in a large number of extra-intestinal infections belong to phylogenetic group B2 and D [4] While phylogenetic groups B2 and D have a lower incidence than A or B1, strains of group B2 survive longer than the other groups. Besides, *E. coli* strains of phylogenetic group B2 and D are more isolated from extra-intestinal sites than group A or B1 strains. Some strains of phylogenetic group B1 persist in water environments. The relationship between virulence and phylogenetic groups of *E. coli* has been reported in many cases. Therefore, identification of unknown *E. coli* strains may provide important information about their physiological and ecological features [5].

Multiple techniques can identify phylogenetic groups including Multilocus Enzyme Electrophoresis (MLEE), ribotyping, random amplified polymorphic DNA analysis, Fluorescent

Amplified-Fragment Length Polymorphism (FAFLP) analysis, PCR phenotyping using the presence/absence of three genomic DNA fragments, analysis of variation at mononucleotide repeats in intergenic sequences, and Multilocus Sequence Typing (MLST). The best technique is MLST, but it is expensive and time-consuming. PCR phylotypic technique is based on a triplex PCR that used two genes chuA and yjaA and TSPE4.C2 DNA fragment. The results of this method strongly correlate with results obtained by other standard methods. So, it is a preferred technique for rapid and cost-benefit phylogenetic identification of *E. coli* strains [6].

Multiplex PCR method (simultaneous amplification of multiple genes) takes the advantage of two or more primer pairs designed for pathogen-specific unique sequences within a single reaction allowing the simultaneous amplification of more than one target sequence by the aid of multiple sets of oligonucleotides to amplify two or more favorable targets. This method permits the concurrent detection of several food borne pathogens. For example, simultaneous detection of *E. coli* O157:H7, Salmonella spp. and *S. aureus*. Advantages of multiplex PCR include significant amplification of multiple targets without any need to extra time and cost and sample volume; however, the competition between oligonucleotide pairs and the subsequent reduction in PCR sensitivity is the drawback of multiplex PCR method [7].

Notably, *E. coli* is one of the most antimicrobial-resistant bacteria and the antimicrobial resistance is an increasing problem worldwide [8].

Meshkin Shahr city is supplied with two distinct water supplies (Khiav Chayi River and Nahr Khatoon) and concerning the importance of water and its contamination and the lack of previous molecular investigations in this field, we decided to examine the potable water supplies of Meshkin Shahr to determine coliforms count and phylogenetic groups of *E. coli* strains.

Material and Methods

Sampling

The water source of Meshkin Shahr, Iran is provided from the Khiav Chayi and the Nahr Khatoon river. In this cross-sectional study, 30 samples were collected from each supply under sterile conditions at different times from 2016 through 2017 to determine the presence of *E. coli* and coliform. All samples transported to the laboratory immediately under the standard condition and processed within 24 hours.

Isolation of *E. coli* by Most Probable Number (MPN) method

This method was carried out with 15 test tubes including three sets of 5 tubes which contain 10 cc lactose broth medium and Durham's tube is inserted in each test tubes. All test tubes were incubated at 37°C for 24 hours. The test tubes were observed after 24 hours to examine gas production and color change. The incubation period was prolonged for 24 hours more if no gas was produced. Positive lactose broth tubes were sub-cultured in *Escherichia coli* broth and incubated at 44.5°C for 24 hours [9,10].

Cultivation on Eosin Methylene Blue (EMB) medium

Positive samples on EC broth transferred to the steak plate. Incubation was done at 37°C for 24 hours. After purification, Indole, Methyl red, Voges-Proskauer, and Citrate (IMVIC) differential tests were conducted for testing colonies. Regarding the results of differential tests, the presence or absence of *E. coli* was determined.

IMVIC test

This test includes four tests. Indole, Methyl Red, Simmon Citrate tests and Voges-Proskauer (VP) test that we used to identify bacterial species.

DNA extraction from *E. coli* strains

At the next step, DNA was extracted from the pure cultures of the bacteria using SinaGen DNA Extraction Kit cat.no:pr881613. Then the extracted DNA was measured by using NanoDrop to ensure its purity. The most proper size is 1.8- 2.2. The produced genomic solution was used in PCR reaction or stored in 4°C for further research.

E. coli has an enzyme called Gad, which converts glutamic acid into α-amino butyric acid. *E. coli* was identified using a 373 Kb GAD gene fragment according to Table 1. For confirm the accuracy of the identity of *E. coli* by using this sequence, all extracted DNA was amplified by PCR separately then were electrophoresed on 2% agar gel and finally if Considered DNA bands appear *E. coli* is confirmed.

PCR optimization

To find proper concentrations of reactants and annealing optimum temperature to reach the acceptable product, we tried multiple tests for each parameter and finally used the following condition to come in Table 3.

Identify phylogenetic groups of *E. coli*

1.5 μL of each primer pair was mixed with 1 μL of DNA and 25 μL of master mix to conduct PCR reaction. Then was added distilled water to make 50 μL total volume of sample and the reaction was carried out in 30 cycles. Gel electrophoresis was done to identify chuA, yjaA genes and TPSE4.C2 DNA fragment in PCR products. The DNA-free sample was used as a negative control. Gel electrophoresis was done on 2% agarose gel as follows: 5 μL of DNA ladder was transferred into one well and our samples containing PCR product and dye in 5:1 ratio respectively loaded to other wells. We used another well as a negative control. Gel electrophoresis was conducted with 80 mV for 45 minutes. The identification of resultant bands of Electrophoresis was performed as compared to the standard molecular weight marker (ladder). Gel imaging was done by Gel Doc™1000 fluorescent imaging system under ultraviolet light. After electrophoresis, the strains were

Table 1: Nucleotide sequence of gad gene.

Primer	Primer sequence (5´- 3´)	Product Length
373	GATGAAATGGCGTTGGCGCAAG	gadA (F)
373	GGCGGAAGTCCCAGACGATATCC	gadA (R)

For cloning of *E. coli* gad gene two different primers with same product length have been used.

Table 2: Primers used to detect bacterial DNA.

Amplicon size (in bp)	Primer sequence (5´- 3´)	Primer
281	ATGATCATCGCGGCGTGCTG	chuA (F)
281	AAACGCGCTCGCGCCTAAT	chuA (R)
216	TGTTCGCGATCTTGAAAGCAAACGT	yjaA (F)
216	ACCTGTGACAAACCGCCCTCA	yjaA (R)
152	GCGGGTGAGACAGAAACGCG	TSPE4.C2 (F)
152	TTGTCGTGAGTTGCGAACCCG	TSPE4.C2(R)

chuA, yjaA and TSPE4 are 3 fragments for detect *E. coli* DNA. Each fragment has 2 different sequences in our study.

Table 3: PCR conditions.

Time	Temperature	Stage
4 min	94°C	Initial denaturation
30 s	94°C	Denaturation
30 s	65°C	Annealing
30 s	72°C	Extension
5 min	72°C	Final Extension
Cycle Number (30)		

After some trial and error we found this optimal PCR conditions which we used for our study.

Table 4: Phylogenetic groups of *E. coli* using chuA, yjaA and TSPE4.C2.

Genes	Phylogenetic groups						
	B2	B2	D	D	B1	A	A
chuA	+	+	+	+	-	-	-
yjaA	+	+	-	-	-	+	-
TSPE4.C2	+	-	+	-	+	-	-

Phylogenetic groups were determined by 3 fragment chuA, yjaA and TSPE4.C2. The strains negative for all fragments and the strains which just positive for yjaA were group A. The strain positive for all fragments and the strains which positive for chuA and yjaA and negative for TSPE4.C2 belong to group B2. The strains positive only for TSPE4.C2 were group B1. The strains negative for yjaA and positive for chuA belong to group D.

categorized based on the presence/absence of reference genes as shown in Table 4.

Antimicrobial susceptibility test

Antimicrobial susceptibility was performed by using Kirby-Bauer method on Mueller-Hinton agar plates for 14 antibiotic agents as fallow ampicillin (AMP) 10 μg, penicillin (PEN) 10 units, cefotaxime (CTX) 30 μg, cefuroxime (CXM) 30 μg, Pipemidic Acid (PA) 20 ug, chloramphenicol (CHL) 30 μg, ciprofloxacin (CIP) 5 μg, co-trimoxazole (COT) 25 μg, erythromycin (ERY) 15 μg, nitrofurantoin (NIT) 300 μg, nalidixic acid (NAL) 10 μg, amikacin (AMK) 30 μg, gentamicin (GEN) 10 μg and tetracycline (TET) 30 μ.

Antimicrobial susceptibility was confirmed according to CLSI guidelines. We considered the intermediate susceptibility as resistant because of its tendency to resistance.

Statistical analysis

Spss software version 16 was used for statistical analysis. All data were analyzed using chi-square and t-test for group comparisons. Significance was attributed when $P<0.05$.

Results

60 samples were collected randomly at different times from two water supplies in Meshkin Shahr, Iran in a year. All 60 samples were evaluated of the presence of total coliforms. Total Coliform contamination load was 620.467 cfu in 100 ml for Kiav Chayi and 92.750 cfu in 100 ml for Nahr Khatoon.

All coliform positive samples were tested for fecal coliforms and 57 samples of 60 coliform positive samples were fecal coliform positive too. Average fecal coliforms contamination loads for Khiav Chayi and Nahr Khatoon were 497.8242 cfu in 100 ml and 66.703 cfu in 100 ml of samples respectively as shown in (Table 5).

t-test results for the correlation between coliform count and Khiav Chayi and Nahr Khatoon samples with a *p*-value of zero were considered as meaningful with CI of 312.4907- 742.9427. t-test results for the correlation between fecal coliform count and Khiav Chayi and Nahr Khatoon samples with a *p*-value of 0.001 were considered as meaningful with CI of 153.2855- 524.8279.

Table 5: Statistical analysis of coliforms and fecal coliforms (cfu /100ml).

Souces	Total coliforms	Fecal coliforms
Khiav Chayi	620.467±576.7983	405.760±497.8242
Nahr Khatoon	92.750±118.8467	66.703±102.7576
Total	356.608±491.1950	236.232±395.2610

Data are mean ± standard deviation.

Table 6: Identification of the isolated bacteria through IMVIC test.

Source	IMVIC		Total
	Other Entrobacteriaceae	*E. coli*	
Khiav Chayi	16	14	30
Nahr Khatoon	9	18	27
Sum Total	25	32	57

According to IMVIC test isolated bacteria classified to *E. coli* and other entrobactriaceae.

Table 7: The frequency of phylogenetic groups.

Sample source	Phylogenetic Group					Total
	A	B1	B2	D	NON	
Khiav Chayi	6	5	1	0	2	14
Nahr Khatoon	1	12	0	2	3	18
Total	7	17	1	2	5	32

Data show the number of phylogenetic group in water sources.

A t-test was used to compare total coliform count in Khaiv Chay and Nahr Khatoon which was significantly different (*P*-value: 0). Also, fecal coliforms deference in two water supply was significant (*p*-value 0.001).

Results of IMVIC test

From 57 samples isolated after cultivation on EMB medium and tested by IMVIC biochemical, 32 *E. coli* samples (56.14%), 21 Citrobacter samples (36.84%), 3 Salmonella samples (5.26%), and one unknown sample were determined (Table 6). Chi-square analysis revealed that the number of *E. coli* bacteria isolated from Nahr Khatoon was higher than Khiav Chayi but there was no significant relationship between *E. coli* count and water supply (*p*-value:0129).

PCR was performed using exclusive primers for gad gene amplification. All samples were *E. coli*-positive.

PCR results and determining phylogenetic groups

After gel electrophoresis in the presence of TSPE4.C2, yjaA and chuA (Figure 1), we found that of 32 confirmed *E. coli* samples, 17 samples (53.12%) belong to phylogenetic group B1, one sample (3.1%) belong to group B2, 7 samples (21.87%) were categorized in group A, two samples (6.25%) belong to group D, and 5 samples (15.62%) were categorized in the unknown group as shown in (Table 7).

By analyzing the relationship between polygenetic groups and water supplies with Chi Square test we realized there was no meaningful relationship between them (*p*:0.054).

Result of antimicrobial susceptibility test

61% of the *E. coli* isolates were resistant to at least one antibiotic. Resistance to ampicillin was the most common among other antibiotics. 95% *E. coli* isolates were resistant to ampicillin, 47% to cefotaxime, 41% to cefuroxime, 90% to penicillin, 22% to gentamicin,

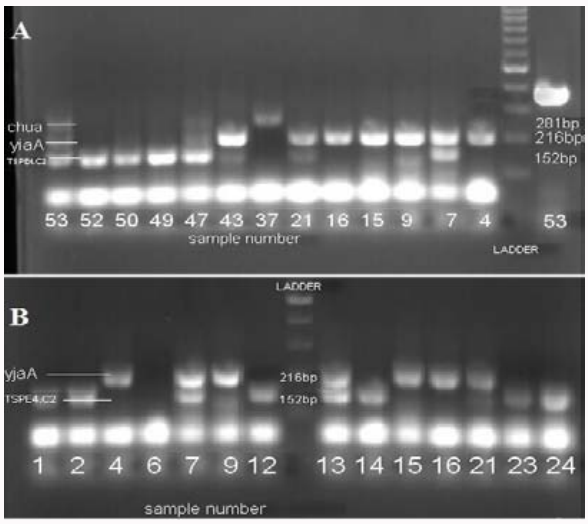

Figure 1: Horizental numbers refer to the samples.The ladder is appropriately labeled to illustrate relative band sizes. Results in amplicons of 152, 216 and 281 bp respectly refer to TSPE4.C2, yjaA and chua.

72% to chloramphenicol, 18% to ciprofloxacin, 79% to erythromycin, 63% to tetracycline, 52% nalidixic acid, 41% to nitrofurantoin, 68% to co-trimoxazole,14% to amikacin, and 57% to pipemidic acid.

Our study showed 33% isolates were resistant to more than one antibiotic and classified as Multi Drug-Resistant (MDR). All isolates in MDR group were resistant to ampicillin. Most resistant isolated *E. coli* belong to subgroup B2 (34%) but this difference between phylogenetic subgroups was not significant.

There was no significant difference in antimicrobial resistance between *E. coli* isolates obtained from to water supplies (*p*:0.2).

Discussion

Water is essential for life and its importance is obvious in drinking, hygienic, agricultural, and industrial usages, Increase in population, urbanization, and industrial and agriculture growth led to increased water consumption and the generation of the sewage which contaminate the environment. The water resources have been seriously threatened by human communities and industrial facilities. In Iran, rivers are critical resources with different uses. These resources usually overuse and there isn't a proper plan for usage of these resources especially in Iran. Khiav Chayi and Nahr Khatoon Rivers had played an important role as vital water supplies for years, particularly as agricultural and drinking water resources. Unfortunately, the mismanagement of these resources use and contamination with nomadic or rural community's wastes and water waste of spa and public pools even contamination with passenger trash degraded the quality of these water supplies and increased its pollution. Regarding the importance of Khiav Chayi and Nahr Khatoon rivers in the agriculture and because of the importance of these water supplies in providing potable water and for realization accurate quality of these supplies, we decide to investigate microbial contamination of these resources.

Direct or indirect contamination with human or animal feces is one of the most common problems for surface water resources which can cause illness after drinking or preparing food or even after skin contact. At present, besides physicochemical parameters, quality control of potable water includes microbial contamination based on total fecal bacteria count, coliforms and *E. coli* is necessary [11,12].

The prevalence of *E. coli* in our study was 56.1% which was close to the result of the study done before by Soltan Dallal et al., He used multiplex PCR as we used for study for evaluate the well waters of Tehran parks and find that 54.5 percent of samples were contaminated by *E. coli* [13].

The results of our study showed that 21.8% of strains belong to group A and 53.1 % were in the phylogenetic groups of B1 which is consistent with the research of Obata-Yasuoka et al.

Obata-Yasuoka M found that about 64% of groups isolated from Jeonnam Province, South Korea belong to groups A and B1 [14]. There are other studies which confirm our finding [15-17] while some studies show those abundant groups belong to B2 [18-19]. We think this difference could be originated from a different diet, antibiotic consumption, and/or different geographic climate [20].

Group B1 was the common group in our study and we know that is abundant in herbivorous hosts However we cannot conclude that these water pollution could be related to these animals because B1 has prolonged survival ability than other strains so it is obvious to find it more in our cultures and also this group could be found in other hosts too [3].

Our findings show that *E. coli* has the highest resistance to ampicillin as some previous studies found that too [21-23]. Also, the prevalent use of antibiotics in medicine and agriculture is known as a common cause in the high occurrence of antibiotic resistance among gram-negative bacteria [24].

In Iran regardless of government control almost any drug especially commonly used antibiotics like ampicillin and penicillin can be acquired in drugstores without the need for a medical prescription [25-27] so self-medication can explain the high prevalence resistance to these antibiotics in our study. On the other hand, according to previous studies, our findings showed a progressively rising rate of antimicrobial resistance in *E. coli* [28] and it could be a global concern.

Conclusions

We realized that Nahar Khaton has lower coliform contamination than Khiav Chay so it could be a better resource for use. Also, we concluded that because of antimicrobial high resistance in isolated *E. coli* the analysis of the water coliform especially determine of *E. coli* and its phylogenetic groups and antimicrobial susceptibility in the water supply can be useful as a prediction tool for planning to protect the water supply and treat it.

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