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Molecular characterization of virulence genes and antibiotic resistance among fecal *Escherichia coli* isolated from surface water of Wadi Shueib-Jordan

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Abstract

Objective: Contamination of surface water with pathogenic organism is highly dangerous for people who used it for drinking or for domestic activities. Detection of *Escherichia coli* in water can be used as a general important indicator of fecal contamination. This study investigated the occurrence of fecal *E. coli*, two important toxigenic types of *E. coli* isolates and their antimicrobial resistance in water samples collected from the surface running source of Wadi Shueib in Jordan.

Methods: A total of 51 water samples were collected from three different locations of wadi shueib over a three month, July through September, 2016. For each sample, 200 ml of water was collected in sterilized containers. All samples which were positive for fecal *E. coli* were subcultured on Eosin Methylelne Blue Agar and incubated at 37 °C for 24 hours. *E. coli* isolates were identified by API- 20 E test, and all isolates were tested for antimicrobial susceptibility and for the presence of virulence genes of enterotoxigenic *E.coli* (ETEC) and enterohemolytic E.coli (EHEC).

Results: A total of 46/51 (90%) of water samples were contaminated with fecal *E. coli*. The 46 *E. coli* isolates were resistant in the range between 4%-76% to commonly used antibiotics in the treatment of infection in Jordan. Multidrug resistant isolates to at least three antibiotics accounted for 17/46 (37%) of the isolates. Out of 46 fecal *E. coli* isolates, 4 (8.7%) were ETEC and 2 (4.3%) were EHEC as detected using PCR.

Conclusion: This study indicated that the surface running water of Wadi Shueib is contaminated with potential enteropathogenic *E. coli*,

and it can be a source of causing diarrheal diseases. Fecal *E. coli* isolates from water also showed high level of antibiotic resistance comparable to human *E. coli* isolates in Jordan.

Keywords
Fecal *E. coli*; ETEC; EHEC; Antimicrobial Resistance; Surface Water; Jordan.

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Introduction

The quality and availability of fresh safe water is an essential element in health and living for every human community [1-2]. Water borne infection are still a major public health concern in most developing countries [3].

Jordan is considered as one of the ten most water scarce countries in the world. High population growth and flow of hundred thousands of refugees from other Arab countries, have aggravated the shortness of ground water reserves in the country [4]. Therefore, every drop of water should be considered important for usage in human activities and agriculture [5].

Wadi Shueib represents a draining area of approximately 180 square kilometers (69 sq mi). Towns and villages along the wadi include Salt, fuheis and Mahis. These discharge treated and untreated sewage into wadi Shueib course. Therefore, high load of biological contaminants, especially waterborne microbial pathogens, would be expected.

E. coli is the most commonly used indicator of fecal contamination in drinking water distribution systems. *E. coli* of enteric origin can persist with other enteric bacteria and parasites for long time in water, and can be usedt as indicator of water contamination [6]. *E. coli* survives in drinking water between 4 and 12 weeks, depending on many environmental conditions [7].

Antimicrobial resistance genes are widely distributed in human intestinal bacterial flora, particularly in gram negative enteric bacteria contaminated aquatic environment [8]. In water, bacteria of different human and animal origins are able to mix with environmental commensal bacteria and contribute for transfer of antibiotic resistance genes [9]. In recent years, infections caused by multidrug resistant (MDR) pathogenic bacteria have severe health implication in patients and offer limited treatment options [10]. Many diarrheagenic *E. coli* types can be also associated with contamination of water, particularly ETEC, EHEC and enteropathogenic *E. coli* (EPEC) [11]. The implementation of PCR methods can easily and rapidly identify diarrheagenic *E. coli* in water and human stool samples [12].

This study aimed to investigate the general occurrence of *E. coli* and particularly the presence of enterotoxigeneic and entereohemorrhagic *E. coli* in water of Wadi Shueib and their antibiotic resistance profiles.

Material and Method

Water samples collection

Water samples were collected from three different locations of Wadi Shueib; water springs before Al-Salt wastewater treatment plant, treated water immediately after Al-Salt wastewater treatment plant (WWTP) and final region of surface water before Wadi Shuaib Dam. For each sample, 200 ml of water was collected in sterilized containers (glass bottles).

When the sample was collected, an ample air space was left in the bottle (2.5 cm) to facilitate mixing and shaking before examination. Bottles were fully submerged in the water, then immediately covered by the sterilized lid according to the guidelines of the American Public Health Association [13]. The samples were carried in ice box and brought to the laboratory within few hours for microbial analysis.

Isolation of fecal E.coli

Out each water sample, 1 ml of water was taken and delivered to Lauryl Tryptose broth (Neogen, USA) with inverted durhams tubes, then incubated at 35°C for 24 hours, and examined for gas formation or acidic growth. When no gas or acidic growth had formed, inoculated tubes were re-incubated for another 24 hours. To detect fecal *E. coli*, positive Lauryl Tryptose broth tubes were shaken to resuspend the organism, and with a sterile loop, one loopful of culture was transferred to EC Broth (HiMedia, India) and incubated at 44.5 °C for 24 hours and examined for growth and gas formation [13]. Test tubes which show positive results were subcultured on Eosin Methylelne Blue (EMB) Agar (HiMedia, India), and were identified by API- 20 E test. *E. coli* isolates were stored in Brain- Heart Infusion broth (Oxoid, England) with 15% glycerol in freezer for further examination.

Antibiotic susceptibility test

E. coli isolates were tested by disk diffusion method against the following antimicrobials (Oxoid, England): Ampicillin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), tetracycline (30 µg) cotrimoxazol (25 µg) amikacin (30 µg), piperacillin-tazobactam (100/10 µg), impeneme (10 µg), meropenem (10 µg), azetreonam (30 µg), and ceftazidime (30 µg) [2,14]. Antimicrobial susceptibility testing was performed according to the recommendation of the Clinical Laboratory and Standards Institute/ CLSI [15]. The results were interpreted according to the guidelines of CLSI. *E. coli* ATCC 25922 strain was included as a control was obtained from Dr. Asem Shehabi, The Jordan University, Amman, Jordan.

Detection and characterization using PCR

All DNA of E. coli isolates and control E. coli strains were extracted using Wizard® Genomic DNA Purification Kit (promega, USA). Control strains of EHEC (ATCC 43894) and ETEC (ATCC 35401) (Dr. Asem Shehabi) were included as positive controls. DNA templates of fecal E. coli strains were subjected to multiplex PCR using specific primer (Integrated DNA Technologies, USA) sequences for the detection of virulence genes in ETEC and EHEC as shown in **Table 1** [16].

Table 1. Primers used in the multiplex PCR for the detection of ETEC and EHEC [16].

Primer	Target gene	Primer sequence	Amplicon size (bp)
LT	eltB	5'-TCTCTATGTGCATACGGAGC-3' 5'-CCATACTGATTGCCGCAAT-3'	322
ST	estA	5'-GCTAAACCAGTAGGGTCTTCAAAA-3' 5'-CCCGGTACAGGCAGGATTACAACA-3'	147
VT1	vt1	5'-GAAGAGTCCGTGGGATTACG-3' 5'-AGCGATGCAGCTATTAATAA-3'	130
VT2	vt2	5'-ACCGTTTTTCAGATTTTGCACATA-3' 5'-TACACAGGAGCAGTTTCAGACAGT-3'	298
eae	eaeA	5'-CACACGAATAAACTGACTAAAATG-3' 5'-AAAAACGCTGACCCGCACCTAAAT-3'	376

In addition to the primers, the mixture contain 12.5 µl master mix (Promega, USA), 2 µl purified DNA and a 0.2µM concentration of each primer except primer VT1 (which was used at a concentration of 0.4µM). PCR reactions were performed in a total reaction volume of 20 µl. The amplification conditions were as the following: 96°C for 4 min, 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s for 30 cycles, with a final 7-min extension at 72°C. 10 µl of each PCR product was evaluated with a 1.5% (wt/vol) agarose gel (Bio Basic Inc., USA) stained with ethidium bromide. Agarose gel electrophoresis was run for 30 min at 120 V [16]. A 50 bp ladder (iNtRON Biotechnology, USA) was used as control.

After electrophoresis was completed the gel was visualized under ultraviolet light.

Results

Incidence of fecal *E. coli* in water

In order to evaluate the presence of fecal *E. coli* in water, a total of 51 water samples were collected from Wadi Shueib. The water samples were examined using a protocol for the presence of fecal *E. coli* and the results showed that 46 samples (90.2 %) showed contamination with fecal *E. coli* **(Table 2)**.

Table 2. Distribution of fecal *E. coli* in water samples collected from Wadi Shueib.

Collection site of water sample	no. of samples	No. of fecal *E. coli* isolates	%
Water springs	17	15	88
Immediately after Al-Salt wwtp	17	16	94
Before Wadi Shuaib Dam	17	15	88

Antimicrobial resistance of 46 fecal *E. coli* isolates

Antimicrobial resistance rates of *E. coli* isolates are shown in Table 3. Out of 46 *E. coli* strains, 17 (37%) isolates were resistant to at least three antibiotic classes and considered multidrug resistant (MDR) isolates[17]. Most of these MDR isolates were isolated from water springs **(Table 3, 4)**.

Incidence of ETEC and EHEC isolates

Out of 46 fecal *E. coli* isolates, 4 (8.7%) were ETEC and 2 (4.3%) were EHEC as detected using PCR **(Table 5)**.

Discussion

This study demonstrates that the surface water of Wadi Shuaib is highly contaminates with fecal *E. coli*, since 46/51 (90.2%) of the water samples were found contaminated with fecal *E. coli*. The isolates were resistant in the range 4%-76% to commonly

Table 3. Resistance rates of 46 fecal *E. coli* isolates to antimicrobial agents.

Antimicrobial agent	No. of resistant isolates	%
Ampicillin (AM)	35	76.1
Tetracycline (TE)	19	41.3
Streptomycin (S)	10	21.7
Cotrimoxazol (STX)	12	26.1
Nalidixic acid (NA)	8	17.4
Azetreonam (ATM)	4	8.7
Ceftazidime (CAZ)	4	8.7
Ciprofloxacin (CIP)	4	8.7
Cefotaxime (CTX)	2	4.3
Gentamicin (CN)	2	4.3
Amikacin (AK)	0	0.0
Impeneme (IMI),	0	0.0
Meropenem (MEM)	0	0.0
Tazobactam (PTZ)	0	0.0

Table 4. Distribution of MDR *E. coli* isolates in water samples collected from different location along the water flow.

Collection site of water sample	no. of samples	No. of MDR *E. coli* isolates	%
Water springs	15	8	53.3
Immediately after Al-Salt wwtp	16	3	18.8
Before Wadi Shuaib Dam	15	6	40.4

Table 5. Distribution of virulence genes in 4 ETEC and 2 EHEC isolates from different location of the surface water of Wadi Shuaib.

Collection sites	no. of *E. coli* isolates	ST	LT		vt1		vt2	eaeA	
			No.*	%	No.*	%	No.*	No.*	%
Water springs	15	0	1	6.7	0	0.0	0	0	0.0
After Al-Salt wwtp	16	0	0		1	6.3	0	1	6.3
Before Wadi Shuaib Dam	15	0	3	20.0	1	6.7	0	1	6.7
Total	46	0	4	8.7	2	4.3	0	2	4.3

*: Pathogenic *E. coli* strains carrying virulence genes.

used antibiotics in the treatment of infection in Jordan [8, 18]. A total of 17 (37%) *E. coli* isolates were multidrug resistant to three or more antimicrobial classes.

A previous study from Saudi Arabia [2] found that *E. coli* isolates from Al-Ahsa water springs exhibited high resistance rates as found in our study for the following antimicrobial agents: tetracycline (50%), cotrimoxazol (38.5%), streptomycin (65.4%), nalidixic acid (42.3%), and ciprofloxacin (34.6%). In addition, the percentage rate of multiresistant strains were higher (57.7%) than this study (37%). The important aspect of contamination of running surface water with opportunistic pathogenic bacteria constitutes the occurrence of antibiotic-resistant bacteria of human and animal sources into aquatic environment. Antibiotic resistance genes are introduced in natural bacterial ecosystems through mainly contamination of water with fecal bacteria. In such systems, communsel bacteria in water could acquire antibiotic resistance genes through natural genetic processes such as conjugation and transduction [19]. This study shows that *E. coli* isolates from water samples carried antibiotic resistance profiles comparable to *E. coli* isolates from human sources in Jordan [18, 19].

Diarrhea caused by pathogenic types of *E. coli* is still a health care problem in developing countries including Jordan, mostly found in children younger than five years old [11, 20, 21]. Among diarrheagenic bacteria, enterotoxigenic and enterohemorrhagic *E. coli* are important enteropathogens causing single and outbreaks of diarrhea following consumption of contamination of fresh food and drinks [22]. ETEC affects small intestine, and it is still a major cause of traveler diarrhea. It is responsible for 280 million diarrheal episodes and more than 400 thousand death annually [23]. ETEC strains produce one or two types of enterotoxins; heat-stable enterotoxin and heat-labile enterotoxin, encoded by the *estA* and *eltB* genes, respectively [24]. While EHEC are more associated with sever outbreak of intestinal infections, and its enterotoxins affect large intestine and leads to severe abdominal pain, watery diarrhea followed by bloody diarrhea and may cause fetal hemolytic uremic syndrome in certain patients. EHEC strains produce verocytotoxins VT1 and/or VT2, which are encoded by the *vt1* and *vt2* genes, respectively [24]. This study demonstrates that ETEC (8.7%) is frequently more detected in the surface water than EHEC (4.3%). The higher incidence of ETEC isolates has also been reported previously in a study performed in Jordan and which has showed that children with diarrhea and without diarrhea carried in their feces mostly EPEC and ETEC [21]. On the other hand, another study in Jordan carried in 2012 showed that non of *E. coli* isolates which contaminated green leafy vegetables, belonged to diarrheagenic *E. coli* [14]. The higher occurrence of the ETEC type than EHEC strains suggests that human fecal material is the main source of contamination of water sources with fecal *E. coli,* since EHEC found mostly in feces of animals [25]. Additionally, heat-stable toxin gene (ST gene) is generally found in ETEC of animal origin, while the heat-labile toxin gene (LT gene) is more detected in human isolates (8.7%) as the case in our study [21].

Conclusion

This study shows that the surface running water of Wadi Shueib is contaminated with potential enteropathogenic strains of *E. coli*, and this organism can be associated with other potential pathogenic organisms and a source of causing diarrheal and enteric diseases. In addition, fecal *E. coli* isolates from this Jordanian surface water has showed high percentage of antibiotic resistance.

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