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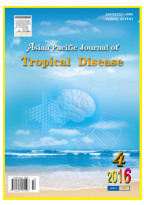

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Investigation on prevalence of *Escherichia coli* strains carrying virulence genes *ipaH*, *estA*, *eaeA* and *bfpA* isolated from different water sources

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ABSTRACT

Objective: To investigate prevalence of *Escherichia coli* (*E. coli*) strains carrying virulence genes *ipaH*, *estA, eaeA* and *bfpA*, isolated from different water sources in Alborz Province.

Methods: This study was carried out in 2014. The research included all *E. coli* strains isolated from different surface water sources in Alborz Province of Iran. *E. coli* isolates were detected and identified by standard microbiological and biochemical tests. The strains were evaluated for the presence of virulence genes *ipaH*, *estA*, *eaeA* and *bfpA* by PCR using specific primers. The PCR amplicons were visualized via electrophoresis and stained with ethidium bromide.

Results: One hundred *E. coli* strains were isolated and included in the study. The PCR results showed that 97% of the strains harbored *ipaH* gene. Moreover, *estA*, *eaeA* and *bfpA* genes were found in 37%, 31% and 3% of the isolates.

Conclusions: Our finding showed that the prevalence rates of virulence genes *ipaH* and *estA* were very high among *E. coli* strains isolated from different surface water sources in Alborz Province. Considering their plasmid-borne nature, the risk of transmission of these genes between other bacterial species could pose a high threat to public health.

1. Introduction

Escherichia coli (*E. coli*) is common and natural inhabitant in the intestinal tract of warm-blooded animals, including humans. *E. coli* associated diseases and water-borne outbreaks result in high morbidity and mortality worldwide. This bacterium most commonly found in food or water is generally considered to directly or indirectly indicate fecal contamination and the possible presence of enteric pathogens[1-3]. The presence of fecal contamination in creeks, rivers, and lakes can lead to the degradation of water quality and subsequently, the water becomes unfit for potable/non-potable uses, aquaculture, and recreational activities such as fishing and swimming[4-7].

Water is necessary for crop production and used for irrigation, freeze protection, pesticide applications, and other agricultural purposes[8]. Surface water sources are considered to be at high risk for pathogen contamination because they are open to numerous routes via which microorganisms causing plant disease and human food-borne illness can enter. Human pathogenic bacteria are believed to enter surface waters mainly through contamination from faeces from livestock and wildlife directly or indirectly by contaminated water, debris, or soil[9,10].

E. coli is generally considered as nonpathogenic. However, some specific strains are pathogenic and can cause disease outbreaks

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associated with contaminated food[11], or with recreational waters[12,13] and drinking waters[14]. *E. coli* is a Gram-negative bacillus, a normal microorganism in humans, but can produce symptoms of diarrhea when virulent factors such as enterotoxins, adhesins, and colonization factors are acquired[15-17].

E. coli, a common indicator of water quality, can also be pathogenic and several diarrheagenic pathotypes, such as enterotoxigenic *E. coli* (ETEC), whose most distinctive genes are the stable thermotoxin -*st*- and thermolabile -*lt*-; enteropathogenic *E. coli* (EPEC), whose characteristic genes are the intimin (*eaeA*) and the bundle forming pilus (*bfp*); Shiga toxin-producing *E. coli*, whose toxins are encoded in the *stx1* and *stx2* genes; enteroinvasive *E. coli* (EIEC), one of whose characteristic virulence traits is the *ipaH*, which belongs to the invasion plasmid; enteroaggregative *E. coli*, with the pCVD432 plasmid, for which the *aatA* gene is one of the most stable regions; and diffusely adherent *E. coli*, whose virulence genes have yet to be fully profiled, have been recognized based on the specific virulence genes present[3,18,19] and implicated in many waterborne outbreaks[20-22].

All of the strains are associated with watery diarrhea, but some strains are associated with vomiting (ETEC), fever (EIEC and ETEC) and bloody diarrhea (enterohemorrhagic *E. coli*)[23]. Numerous studies have shown that fecal material from several animals and humans contains *E. coli* carrying virulence genes associated with pathogenic *E. coli*[6,24-26] and can be a potential source of pathogenic *E. coli* in the surface waters. Amid the diarrheagenic pathotypes of *E. coli*, EPEC and Shiga toxin-producing *E. coli* are more frequently associated with global waterborne outbreaks[20,22,27,28].

Contamination of surface waters with pathogenic strains of *E. coli* has been implicated in increasing number of disease outbreaks and deaths[29,30]. Disease outbreaks related to exposure to contaminated freshwaters are well documented[30-33]. The rate of pathogenic *E. coli* strains harbouring virulence genes in environmental waters could be linked to contamination by storm events, faeces from wild and domestic animals as well as humans, runoffs from agricultural lands, sewage overflows, farm animals, pets and birds[6,34-37]. However, only some studies have investigated the presence of *E. coli* strains carrying virulence genes in environmental waters[7,38-43]. To the best of our knowledge, no investigation on *E. coli* virulence gene distribution has been carried out in the different water sources in Iran. Therefore, the main aim of the current study was to investigate the prevalence and distribution of *E. coli* carrying virulence genes from surface waters in Alborz Province, Iran.

2. Materials and methods

From September 2013 to September 2014, an overall of 100 different water sources samples were randomly collected from various parts of Alborz Province, Iran. The sterile glass bottles comprising 0.5 g of sodium thiosulphate for dechlorination of the water were used for samples collection. To minimize the risks of contamination, all samples were collected 30 s after opening of faucet. Water samples were immediately moved to the laboratory in cooler with ice-packs. The water samples were collected with particular attention to prevent any contamination during various phases of sampling.

Analysis of the water samples was completed within 2 h after collection via standard multiple-tube lactose fermentation method. Followed by calculating the most probable number, the tubes showing growth were inoculated onto 5% sheep blood and MacConkey agar (Merck, Germany) and incubated for 18 to 24 h at 37 ℃. Colonies with the typical color and appearance of *E. coli* were picked and streaked again on blood agar plates and re-streaked on eosin methylene blue agar (Merck, Germany). The green metallic sheen colonies were considered as *E. coli*. After 24 h incubation at (35.0 ± 0.5) ℃ for (24 ± 2) h, Gram-negative microorganisms were isolated from MacConkey agar and eosin methylene blue agar and determined at the species level using cytochrome oxidase, triple sugar iron agar, urea and indole tests to screen *E. coli* isolates. The stock was kept at –20 ℃ until use. DNA was extracted using AccuPrep® genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. The DNA concentration has been determined by measuring absorbance of the sample at 260 nm using spectrophotometer[44].

The colonies were confirmed using PCR based on the technique previously described[45]. The 10 mL bacterial DNA extract and controls were amplified with 0.5 mmol/L primers (forward: 5'-AGTTTGATCCTGGCTCAG-3' and reverse: 5'-AGGCCCGGGAACGTATTCAC-3') (1 343 bp)[45], 200 mmol/L of each diethyl-nitrophenyl thiophosphate (Fermentas, Germany), 2 mmol/L $MgCl_2$, 10 mmol/L KCl PCR buffer and 1.0 IU of *Taq* polymerase (Fermentas, Germany). The DNA was amplified in a programmable thermal cycler (Eppendorf, Mastercycler® 5330, Germany). PCR device used the following protocol: 94 ℃ for 5 min, 40 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 2 min, and finally 72 ℃ for 5 min.

List of primers and the annealing temperature used for amplification of virulence genes of *E. coli* isolates were shown in Table 1[46]. The PCR amplification products (15 μL) were subjected to electrophoresis in a 1.5% agarose gel in 1× tris/borate/ethylene diamine tetraacetic acid buffer at 80 V for 30 min, stained with ethidium bromide, and images were obtained in a UVItec gel documentation systems (UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany). For each PCR test, corresponding negative (sterile water) and positive controls were

included.

Table 1
Primers used in the PCR.

Target gene	Primer sequence (5'→ 3')	Size of product (bp)	AT (℃)
ipaH	F: GCTGGAAAAACTCAGTGCCT	424	57
	R: CCAGTCCGTAAATTCATTCT		
estA	F: CTTGACTCTTCAAAAGAGAAAATTA	147	60
	R: TTAATAGCACCCGGTACAAGCAGG		
bfpA	F: TTCTTGGTGCTTGCGTGTCTTTT	367	56
	R: TTTTGTTTGTTGTATCTTTGTAA		
eaeA	F: CACACGAATAAACTGACTAAAATG	376	60
	R: AAAAACGCTGACCCGCACCTAAAT		

F: Forward; R: Reverse; AT: Annealing temperature.

3. Results

Among the one hundred confirmed *E. coli* isolates assessed for the prevalence of various virulence genes, 97% harbored at least 1 virulence gene while 3% isolates harboured none (Table 2). The most frequent virulence genes were *ipaH*, *eaeA* and *estA*, each of which was observed in 97%, 37% and 31% of the isolates, respectively. In contrast, *bfpA* were detected in 3% of isolates.

Table 2
Virulence genes of *E. coli* isolated from different water sources in Alborz Province of Iran.

Number of isolates	*bfpA*	*estA*	*eaeA*	*ipaH*
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	-	+	-	+
34	-	+	-	+
35	-	+	-	+
36	-	+	-	+
37	-	+	-	+
38	-	+	-	+
39	-	+	-	+
40	-	+	-	+
41	-	-	+	+
42	-	+	-	+
43	-	-	-	+
44	-	+	-	+
45	-	-	-	+
46	-	-	-	+
47	-	-	+	+
48	-	+	-	+
49	-	-	-	+
50	-	+	+	+
51	-	-	+	+
52	-	-	-	+
53	-	-	-	+
54	-	-	-	+
55	-	+	+	+
56	-	-	-	+
57	-	+	+	+
58	-	+	+	+
59	-	+	+	+
60	-	-	+	+
61	-	-	+	+
62	-	+	-	+
63	-	+	+	+
64	-	+	-	+
65	-	+	-	+
66	-	+	-	+
67	-	+	-	+
68	-	+	+	+
69	-	-	+	+
70	-	-	-	+
71	-	-	-	+
72	-	-	-	+
73	-	-	+	+
74	-	-	-	+
75	-	-	+	+
76	-	-	+	+
77	-	-	+	-
78	-	-	+	+
79	-	-	-	-
80	-	-	+	+
81	-	-	+	+
82	-	-	+	+
83	-	-	+	+
84	-	-	+	+
85	-	-	+	+
86	-	+	+	+
87	-	-	+	+
88	-	-	+	+
89	-	-	-	+
90	-	-	-	+
91	-	-	-	+
92	-	-	-	+
93	-	-	-	+
94	-	-	-	+
95	-	-	-	+
96	+	-	-	+
97	+	-	-	+
98	+	+	-	+
99	-	+	-	+
100	-	-	-	+

(*continued on the right column*)

Table 2 *(continued)*

In the present research, we found that the *E. coli* strains isolated from different water sources carried the virulence-associated *ipaH*, *eaeA* and *estA* genes more frequently. Overall, the virulence gene *ipaH* associated with EIEC strains was the most prevalent (97%) and low prevalence of the *bfpA* gene (3%) was detected. In addition, the prevalence of the *estA* gene was greater than that of the *eaeA,* and *bfpA* genes.

Among virulence genes detected, *ipaH* occurrence was significant in isolates from surface water sources.

4. Discussion

The current study investigated the distribution and frequency of *E. coli* isolates carrying virulence genes from different water sources in Alborz Province, Iran. Generally, the mean counts of the presumptive *E. coli* obtained in different water sources were relatively high. *E. coli* has been used extensively as one of the major faecal indicator bacteria due to the previous notion that it has limited survival ability in the environment; however, recent studies have suggested that a number of pedigrees of *E. coli* have adapted and acclimatized within subtropical, tropical, and even temperate regions[47,48]. An earlier study has also reported the occurrence of high numbers of faecal indicator bacteria originating from defective septic systems and grazing animals in surface waters and freshwaters of developing countries[49,50].

In our study, virulence genes were detected in the *E. coli* isolates suggesting the presence of pathogenic *E. coli* strains in different waters sources. A large number of the *E. coli* isolates tested positive for *ipaH* gene. EPEC has been shown to be a major cause of diarrhea in young children[51]. The *eaeA* gene, which codes for intimin protein, was the fourth most prevalent gene in this study (31%). This gene is essential for intimate attachment to host epithelial cells in both the EPEC and enterohemorrhagic *E. coli* pathotypes. Our findings tend to strongly disagree with the previous result of significantly higher prevalence of the *eae* gene (up to 96%) in surface water reported in other studies[33,52]. Typical EPEC strains carry the locus of enterocyte effacement pathogenicity island, which encodes for several virulence factors, including the plasmid-encoded bundle forming pilus (*bfp*) and intimin (*eaeA*) which mediate adhesion to intestinal epithelial cells[7]. Therefore, all the *E. coli* isolates were further screened for the presence of the *eaeA* and *bfpA* genes to determine their association with the EPEC pathotype.

In current study, a unusually low prevalence of the *bfpA* gene (3%) was detected, suggesting that prevalence of the EPEC-like pathotype could be expected in the surface water bodies. Another important finding is that *eaeA* was also detected in 31% isolates which do not have other typical genes from both EPEC groups. This indicates the prevalence of this gene in *E. coli* isolated from the different water sources. This could indicate the presence of atypical ECEP varieties in the country, which is congruent with findings worldwide, where the atypical EPEC varieties are more frequent than typical ones[53]. This finding is of great concern, as an atypical EPEC pathotype which lacks the *bfpA* gene but carries the *eaeA* gene has been found to be a major cause of gastroenteritis worldwide[54], in patients suffering from community-acquired gastroenteritis in Melbourne, Australia[55], and in children with diarrhea in Germany[56]. The detection of the intimin gene (*eaeA*) in EPEC could indicate the presence of Shiga toxin-producing *E. coli*, since *eaeA* gene is found in most of the EPEC and EHEC pathotypes[57]. The ingestion of EPEC, however, results in watery diarrhea that is associated with low fever and vomiting[23].

Therefore, a better understanding of the distribution and prevalence of *E. coli* virulence genes in water sources used for non-potable, potable, or recreation purposes could be an chief approach in the development of public health risk mitigation strategies. The results showed that the risk of contracting infection may increase over time if no appropriate preventive and controlling measures are ensured. Although the ability of *E. coli* isolates described in this study to cause human diarrhoeal diseases was not established, a high proportion of isolates carrying a full set of virulence genes have been linked to defined pathotypes. Additional screening for other virulence genes along with serotype testing and other assays may offer further information on pathogenicity of these isolates.

The detection of *E. coli* and its virulence genes from surface water sources in Alborz Province, Iran, indicated faecal contamination and the possible occurrence of other enteric pathogens. The prevalence of virulence markers in *E. coli* isolates from different water sources is indicative of increased risks of mortality, therefore, emphasizes the importance of safe water supply, good hygiene and sanitation practices both in rural and urban communities. Finally, this study has revealed a number of *E. coli* isolates positive for single and multiple virulence genes which indicated the presence of potential pathogenic *E. coli* in these waters, and it clearly highlights the need to develop a better understanding of public health implications of occurrence of *E. coli* carrying virulence genes in different water sources used for potable, non-potable, and recreational purposes.

Conflict of interest statement

We declare that we have no conflict of interest.

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