

Spectrum of diarrheagenic *Escherichia coli* in drinking and wastewater in Rafha city of Saudi Arabia

ABSTRACT

Introduction: *E. coli* is one of the most important etiologic agent of diarrhea in children and adults. Based on the clinical features and virulence determinants, there are five major *E. coli* strains which cause diarrhea; Enterohemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), and Enteroaggregative *E. coli* (EAEC). The PCR based identification proves to be a better choice as it can differentiate between different strains on the basis of genetic difference.

Aim: The purpose of the current study was to isolate diarrheagenic *E. coli* from the drinking and wastewater from Rafha city of Saudi Arabia.

Methodology: One 100 drinking and wastewater samples (50 each) were included in the study. The samples were cultured on MacConkey agar plates at 37 °C for 24 hours. Pink colonies were carefully picked and subjected to DNA isolation and PCR detection and identification of *E. coli* and Diarrheagenic *E. coli*. The detected PCR products were sequenced for the confirmation.

Results: We identified 5 isolates out of 50 wastewater samples (10%) which were further categorized into 3 different DEC pathotypes. They included ETEC (2 out of 5), atypical EPEC (1 out of 5), and EAEC (1 out of 5); EIEC and EHEC were not detected.

Discussion and Conclusion: The prevalence of DEC strains is different across different studies which depends on different factors such as geographical location, number of samples taken, and the number and type of the target genes selected. The prevalence of DEC in the current study was much lower than other reported studies. Although the percentage of DEC strains in the wastewater samples from WWTP of Rafha was moderate to low, it can be a considerable risk factor for the people using ground water for drinking.

Keywords: Diarrhea, *E. coli*, Multiplex PCR, Virulence, Pathotypes, epidemiology

Abbreviations: DEC (Diarrheagenic *Escherichia coli*), PCR (Polymerase chain reaction), EAEC (Enteroaggregative *E. coli*), EIEC (Enteroinvasive *E. coli*), EPEC (Enteropathogenic *E. coli*), EHEC (Enterohemorrhagic *E. coli*), STEC (Shiga-toxin producing *E. coli*), ETEC (Enterotoxigenic *E. coli*), WWTP (Wastewater treatment plant)

1. INTRODUCTION

Diarrhea is a major public health concern and is a predominant cause of morbidity and mortality in infants and young children [1]. Developing countries in Asia, Africa and South America are the most affected with diarrheal illnesses resulting in lethal outcomes due to poor living conditions [2]. *Escherichia coli* is a facultative anaerobic, rod-shaped, Gram-negative, coliform bacillus of the genus *Escherichia*, named after the German pediatrician Theodor Escherich and belong to the family *Enterobacteriaceae* [3]. The family *Enterobacteriaceae* is a large family of Gram-negative bacteria which contains many pathogenic and non-pathogenic bacteria such as *Escherichia coli*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Salmonella*, *Shigella*, *Yersinia pestis*, *Proteus* and *Serratia*. The *Escherichia coli* is widely distributed, residing the large intestine of humans and warm-blooded animals [4]. Most *E. coli* strains are non-pathogenic and live friendly in the colon of healthy individuals. However, some strains can be occasionally pathogenic and cause serious food poisoning both in healthy and immunocompromised individuals [5].

There are specific strains of *E. coli* which can cause diarrhea known as diarrheagenic *E. coli* (DEC). Depending on discrete epidemiological and clinical features and precise virulence determinants, the DEC strains are divided into five main types. These types include: enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) or Shiga-toxin producing *E. coli* (STEC), and enterotoxigenic *E. coli* (ETEC). Another newly described type is diffusely adherent *E. coli* (DAEC) which is relatively less well defined subclass. Based on the presence a plasmid containing the EPEC adherence factor (EAF), the EPEC class is further subclassified into typical and atypical strains [6]. Each of these pathogenic subtypes contains specific virulence factors and some subtypes may acquire virulence factors from other pathotypes, becoming possibly more virulent hybrid pathogenic strains [2].

The detection of DEC subtypes is frequently carried out through serotyping, biochemical reactions, virulence factors dependent phenotypic assays and molecular methods especially multiplex polymerase chain reaction (PCR) test [6]. The traditional typing of *E. coli* subtypes is based mainly on two types of surface antigens (Kauffman scheme): LPS (lipopolysaccharide) or O antigens and flagellar proteins or H antigens [7]. The methods for O and H serotyping are tiresome and time-consuming taking 2 to 12 days, not meeting the need for quick diagnosis especially in outbreaks. Also multiple step agglutinations have to be done because there are more than 50 serotypes of H antigens [7, 8]. PCR is a the most frequently used rapid and reliable method that is highly sensitive and specific. A multiplex PCR assay can detect all the pathogenic sub-strains in a single tube reaction [9]. We used multiplex PCR method to detect diarrheagenic *E. coli* in drinking and wastewater in Raha city of Kingdom of Saudi Arabia.

2. MATERIAL AND METHODS

2.1. Sample collection

The study was performed at the Department of basic health sciences, College of Pharmacy, Northern Border University, Rafha city of Saudi Arabia, between February and October, 2018. A total of 100 samples (50 drinking water and 50 wastewater) were included in the study.

The samples were collected in appropriate sterile containers from wastewater treatment plant (WWTP) and public toilets in Rafha and were immediately transported to the laboratory for bacteriological investigation. Fresh drinking water bottles from different companies were purchased from the market.

2.2. Bacteriological analysis

All the samples were cultured onto MacConkey agar plates with the help of sterile swabs and incubated aerobically at 37 °C for 24 hours. Lactose fermenting isolates with red/pink colonies were carefully picked and subjected to DNA isolation and PCR detection and identification of *E. coli* and Diarrheagenic *E. coli*.

2.3. PCR detection of *E. coli*

DNA isolation was done by simply boiling the colonies in 100 µl sterile water for 3 minutes followed by centrifugation for 5 minutes at 5000rpm. The supernatant was separated and used for PCR amplification. For the *E. coli* detection, specific *E. coli* gene segments were amplified by PCR with specific primers using the extracted DNA. The PCR was done in a 200 µl PCR tube. The 20 µl reaction mixture contained 1X PCR buffer (10mM Tris HCL with 50mM KCL, pH 8.3), 2.5 mM MgCl₂, a 0.2mM of each dNTP, 0.2 µM of each of the primers, 0.5U Taq DNA polymerase and 2µl of the DNA template. The sequences of different primers for the detection of *E. coli* used in this study were reported elsewhere in the literature (Table 1). The PCR cycling conditions were; 95°C for 1 min for one cycle followed by 35 cycles of 94°C for 5 seconds, 56°C for 10 seconds, 72°C for 15 seconds. The final extension was done at 72°C for 5 min. The PCR products (6µl) were analyzed using agarose gel (1.5%) electrophoresis (Figure. 1).

Table 1. Primers used for *E. coli* identification

Primer ID	Primer Sequence	Tm	Amplicon Size
lacZ4 F	5'- CTGCTGCTGCTGAACGGCAA 3'	59.5	243
lacZ4 R	5'- CACCATGCCGTGGGTTTCAA 3'	57.5	
M12 F	5'- GTGATCTCCAGCTACCGCTA 3'	57.5	200
M12 R	5'- CGTTGCAAACCTGACGCTCTT 3'	55.4	

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2.4. Multiplex PCR for the identification of different diarrheagenic *E. coli* strains

Multiplex PCR reactions were performed for the identification of each DEC strain; the primers are shown in the Table 2 [10]. The primers were divided into two groups for better amplification and resolution on agarose gel and therefore, two multiplex PCR were performed. The group 1 (annealing temperature 54 °C) contained *eae*, LT, VT2, and ST, whereas the group 2 (annealing temperature 50 °C) contained EA, *bfpA*, SHIG, and VT1 primers. The PCR mix and conditions are described in the previous section. The PCR products were resolved on 1.5% agarose gel and pictures were taken (Figure 1)

2.5. Confirmation by DNA sequencing

The detected PCR products were sequenced for the confirmation of the DNA sequence of the expected DEC strain using Sanger method. The sequencing reactions were done in a BioRad CFX 96 thermal cycler using BigDye Terminator v3.1 kit (Applied Biosystems) according to the manufacturer's protocol. The products of sequencing reaction were precipitated with ethanol and analyzed in an ABI Prism 3130xl genetic analyzer, a 16 capillary sequencer (Applied Biosystems). The sequence trace files were base-called using the Phred program [11, 12] and all low quality bases (<Q20, 99% <accuracy) were eliminated from the sequence ends.

Table 2. sequence of the primers used in the multiplex PCR [10].

Target gene	Primer Name	Primer sequence	Amplicon size (bp)
<i>eltB</i>	LT-F	5'-TCTCTATGTGCATACGGAGC-3'	322
	LT-R	5'-CCATACTGATTGCCGCAAT-3'	
<i>estA</i>	ST-F	5'-GCTAAACCAGTA ^G _A GGTCTTCAAAA-3'	147
	ST-R	5'-CCCGGTACA ^G _A GCAGGATTACAACA-3'	
<i>vt1</i>	VT1-F	5'-GAAGAGTCCGTGGGATTACG-3'	130
	VT1-R	5'-AGCGATGCAGCTATTAATAA-3'	
<i>vt2</i>	VT2-F	5'-ACCGTTTTTCAGATTTT ^G _A CACATA-3'	298
	VT2-R	5'-TACACAGGAGCAGTTTCAGACAGT-3'	
<i>eaeA</i>	eae-F	5'-CACACGAATAAACTGACTAAAATG-3'	376
	eae-R	5'-AAAAACGCTGACCCGCACCTAAAT-3'	
<i>ial</i>	SHIG-F	5'-CTGGTAGGTATGGTGAGG-3'	320
	SHIG-R	5'-CCAGGCCAACAATTATTTCC-3'	
<i>bfpA</i>	bfpA-F	5'-TTCTTGGTGCTTGC GTGTCTTTT-3'	367
	bfpA-R	5'-TTTTGTTTGTGTATCTTTGTAA-3'	
pCVD	EA-F	5'-CTGGCGAAAGACTGTATCAT-3'	630
	EA-R	5'-CAATGTATAGAAATCCGCTGTT-3'	

2.6. The description of the virulence markers was as follows [10]: The *eltB* and/or *estA* are the genes for enterotoxins in ETEC, the *vt1* and/or *vt2* code for Shiga toxins 1 and 2 in EHEC, *eaeA* is a structural gene for intimin found in EHEC and EPEC, the *bfpA* is a structural gene for the bundle-forming pilus of EPEC, the *ial* is the invasion-associated locus of the invasion plasmid found in EIEC and Shigella, and the pCVD is the the nucleotide sequence of the *EcoRI-PstI* DNA fragment of pCVD432 representing EAEC. The minimum criteria for the presence of *eltB* and/or *estA* (ST) for ETEC; the presence of *vt1* and/or *vt2* for EHEC (the additional presence of *eaeA* confirms the detection of a typical EHEC isolate); the presence of only *eaeA* (*eae*) for atypical EPEC; the presence of *bfpA* and *eaeA* for typical EPEC; the presence of *ial* for EIEC and Shigella, and the presence of pCVD (EA) for EAEC.

3. RESULTS

As described in the previous section, 50 wastewater and 50 drinking water samples were included in this study. All wastewater samples yielded bacterial growth on MacConkey agar plates. The isolated pink colonies, at least 5 from each sample plate were picked and subjected to *E. coli* identification through PCR. More than 95% of the selected colonies were detected as those of *E. coli* (Figure 1).

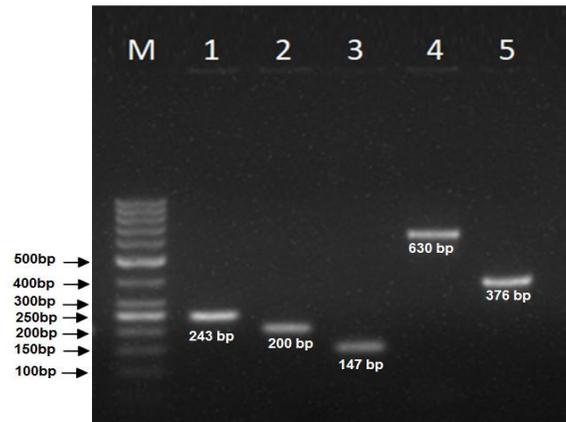


Figure 1. Agarose gel electrophoresis picture showing PCR bands for the detection of *E. coli* and identification DEC strains. Lane M, GeneRuler 50bp DNA ladder (Thermo Fisher Scientific catalogue number SM0371); Lanes 1, *E. coli* LacZ4 gene fragment (243bp); Lane 2, M12 gene fragment (200bp); Lane 3, estA gene fragment (147bp) for ETEC; Lane 4, pCVD gene fragment (630bp) for EAEC; and Lane 5, eaeA gene fragment (376bp) for atypical EPEC.

By using the multiplex PCR for the identification of each DEC strain, we were able to identify 5 isolates out of 50 wastewater samples (10%) which were further categorized into 3 different DEC pathotypes. They included ETEC (2 out of 5), atypical EPEC (1 out of 5), and EAEC (1 out of 5); EIEC and EHEC were not detected (Table 3).

Table 3. The diarrheagenic *E. coli* strains detected in the current study.

Type	Target gene	Primer name	Amplicon size
ETEC	estA	ST	147
EPEC atypical	eaeA	eae	376
EAEC	pCVD	EA	630

For the confirmation of the DEC strains detected by PCR, the PCR products were sequenced in both directions using both forward and reverse primers separately as follows; ST primers for the presence of estA gene (for ETEC), eae primers for the presence of eaeA gene (for atypical EPEC), and EA primers for the presence of pCVD gene (for EAEC). No DEC strains were detected in the drinking water.

4. DISCUSSION

There are five main categories of Diarrheagenic *E. coli* based on the presence of various virulence genes. The detection of these of *E. coli* strains is difficult and time consuming as it requires several PCR reactions. In a study done in Vietnam [10], a multiplex PCR was done using eight primer pairs specific for EAEC, EHEC, EIEC, EPEC, and ETEC in a single reaction. The researchers reported very high sensitivity of these primers for the detection of DEC strains. They further claimed that the reported PCR assay showed positive results for the all the tested DEC strains and negative results for all non-DEC strains, suggesting that the assay was highly specific. We used the primers reported in that study and divided them into two groups for the ease of resolution and detection on agarose gel. In case of performing a single multiplex PCR, the amplified fragments from *eaeA* and *bfpA* (376 and 367 bp, respectively) and from *eltB* and *ial* (322 and 320 bp, respectively) which were quite similar in size and would be difficult to resolve and identify correctly on agarose gel. This would require a separate PCR with specific primers. Therefore, dividing the primers into two sets made it more simple and clear though it increased the labor.

Among all isolated colonies picked for *E. coli* detection, more than 95% tested positive out of which only 5 were DEC strains which shows specificity of the assay. There were no mixed strains as all 5 DEC strains were detected from 5 different samples. Some studies have reported mixed infections from stool samples like two different bacteria or a bacterium and a virus [14, 15, 16, 17]. In some other studies, the presence of different types of DEC strains in a single stool sample have also been reported [10, 15, 18, 19].

The prevalence of ETEC in the current study (6%) was much lower than other reported studies where it was found to be 26% [20]. Higher prevalence was also reported in some other studies [21, 22, 23, 24, 25]. In stool samples taken from diarrhea patients, the prevalence of ETEC was lower (2.2%) in a study done in Vietnam [10] whereas in some other parts of the world, it was shown to be as high as 20.7% [26, 27] and 28% [28]. However, some studies have shown low prevalence of ETEC in children with diarrhea [29].

The percentage prevalence of DEC strains seems to vary across different studies. These differences may be due to different geographical location, number of samples taken, and the number and type of the target genes selected for the study. The percentage of the EPEC positive samples were very low in our study (2%) which is in accordance with the study reported by Mbanga et al., 2020 (1.7 %) [30]. Osińska et al. [31]. however, reported 65% EPEC from raw and processed wastewater from a WWTP in Poland. The prevalence of EAEC in our study was also very low (2%) whereas Mbanga et al., 2020, [30] reported 53.3% prevalence of EAEC in samples taken from WWTP which is even higher than that reported by Osińska et al. [31] 28%.

In a study done by using an 11-gene-single-step multiplex PCR for the detection of DEC in clinical and environmental water samples, the prevalence of DEC strains was as follows; EPEC/EHEC (*eaeA* gene) 40%, EAEC (*eagg*) 35%, ETEC (*lt/st*) 11.3% [32]. In another study conducted in Tunisia [33]

where 60 wastewater samples were taken from 15 WWTPs, the researchers found ETEC was the most prevalent strain (53.3%), followed by EAEC (16.6%), and EIEC (6.6%). The EPEC was not detected in any of the samples. The results of these two studies were much higher than those reported in the current study. However, in another study done in South Africa [34] using samples from two WWTPs, the following prevalence was reported; atypical EPEC/EHEC (7.6%), ETEC (1.4%), and EAEC (7.6%) which are somewhat closer to our results.

5. CONCLUSIONS

Our study is among the few from the region to report the prevalence of DEC in wastewater samples. The results of the current study show that the studied waste water samples from WWTP of Rafha had a moderate to low percentage of DEC strains. Nonetheless, they can contaminate the ground water and there is a considerable risk of getting infected with DEC for the people using ground water for drinking. It can also be a health risk for people working at the WWTP, especially those cleaning the sewage pipelines. Moreover, the study emphasizes the need to closely monitor the health and safety measures for WWTP workers.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. ~~Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors~~ please see the acknowledgment below

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