

## Detection of virulence genes of diarrheagenic *Escherichia coli* strains from drinking water in Khartoum State

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### ABSTRACT

This study aimed to determine the prevalence of virulence genes in all the diarrheagenic *Escherichia coli* DEC strains (EAEC, EHEC, EIEC, EPEC, and ETEC) isolated from drinking water from Khartoum State, Sudan. A total of 46 drinking water samples obtained from different water sources were analyzed for the presence of *E. coli* as fecal contamination indicator and the antimicrobial resistance pattern of isolated *E. coli* DEC strain was investigated. The bacterial genomic DNA was used as a template for multiplex polymerase chain reaction (MPCR) for the detection of ETEC (*stx* gene), EIEC (*ipaH* gene), EPEC (*eae* gene), and EAEC (*aggR* gene) as virulence and biomarker genes. Our results showed that *ipaH* gene was found in 41.3% (19/46) of isolates, and *aggR* gene detected in 30.4% (14/46) of isolates. Both *aggR* and *ipaH* were found positive in 8 (19.5%) isolates and as well the combination of *aggR* and *stx* genes were detected in 2 (4.3%) isolates. In conclusion, this report confirmed the presence of DEC strains in drinking water from different resources and locations. Such findings require separate future clinical research studies to examine waterborne pathogens that exist in this state's water and find a management solution to stop or avoid potential outbreaks.

**Key words** | diarrheagenic, fecal coliform, Khartoum State, multiplex PCR, waterborne pathogen, water-related diseases

### HIGHLIGHTS

- This study reported the presence of DEC strains in drinking water from different resources and locations from Khartoum State, Sudan.
- Such findings require separate future clinical research studies to examine waterborne pathogens that exist in this state's water.
- Find a management solution to stop or avoid potential outbreaks.
- Also, this study reported a high percentage of drug resistance between isolated *E. coli*.
- Sewer can also be considered as a source of transmission of drug-resistant bacteria.

### INTRODUCTION

Diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality in infants and young

children, especially in developing countries (Jafari *et al.* 2012; Liu *et al.* 2012; Fakhr *et al.* 2016). To ensure good health, the drinking water supply must be clean and free of harmful turbidity, dissolved toxins, and waterborne pathogens such as *Giardia*, *Cryptosporidium*, *Campylobacter*, *Salmonella*, *Shigella*, *Vibrio*, *Hepatitis A*, and Norwalk viruses (Okeke

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2009; Cowan & Herzog 2014). Water sources can be investigated for detection of fecal contamination; high fecal levels can mean that water contains pathogens by testing for the presence of *Escherichia coli* (Cowan & Herzog 2014).

*E. coli* is a Gram-negative rod, facultative anaerobe, belongs to the family Enterobacteriaceae, motile by peritrichous flagella, lactose fermenter, oxidase negative, catalase-positive and widely distributed in nature (Ryan et al. 2010). It is found as normal flora in the gastrointestinal tract of humans and animals. A high number of *E. coli* can be shed into the environment via the feces, but most strains are harmless. However, some *E. coli* strains can cause disease, therefore, if it is detected in water, immediate action should be taken to eradicate this contamination, because water-borne pathogens are presented into drinking water supplies via contamination with human or animal feces (WHO 2012). According to their virulence properties, symptoms of the disease that they cause, species and age group where they are found, *E. coli* is classified into: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). The last has been linked to diarrheal disease in various parts of Africa (Okeke 2009; Croxen et al. 2013; Adugna et al. 2015). The most common etiologic agent of diarrhea from water is diarrheagenic *E. coli* (DEC) (Todar 2016; Fakhr et al. 2016), which can be transmitted by fecal-oral contamination (Gomes et al. 2016). DEC strains are differentiated using DNA probes and polymerase chain reactions (PCR) (Brooks et al. 2001; Baylis et al. 2011; Hegde et al. 2012).

Diarrheagenic *E. coli* is defined as some pathogenic *E. coli* strains that can cause diarrhea or extra-intestinal diseases both in healthy and immunocompromised individuals (Gomes et al. 2016). Each strain possesses characteristic virulence factors, for instance, EPEC strain usually performs localized adherence to epithelial host cells through using *eaf* gene/protein. In most cases, this is associated with attaching and effacing lesion formation on enterocytes which are linked to *eae* as virulence genes (Ogata et al. 2002). The O157: H7 serotypes are the important EHEC strains, and *stx* is the essential virulence factor for EHEC, which is also recognized as verocytotoxin (VT) and Shiga-toxin (*stx*) (Baylis et al. 2011). EIEC mainly causes colon infection and has outstanding ability to invade and damage the colonic tissue through the IPaA to

IPaH plasmid invasion antigens (Hill 2013). EAEC generates aggregate adherence mediated by either aggregate adherence fimbriae (AAF/I) or (AAF/II) encoded by *aggR* genes (O'Sullivan et al. 2006). Fecal contamination of drinking water is a major health problem that is responsible for many cases of diarrhea, especially in infants and people from foreign countries (Shanks et al. 2007; Fakhr et al. 2016). Moreover, the DEC infection is becoming more complicated because of increasing antibiotic resistance phenomena among bacterial isolates (Ozgunmus et al. 2007).

While many Sudanese states and cities are experiencing much higher development rates and considerably steeper population growth, Khartoum is witnessing a very different form of urbanization. The state of Khartoum is a patchwork of wealth and poverty, demographic density and widespread and robust and atrophied infrastructure (Abdellah et al. 2014). This results in a large community characterized by crowding, poor sanitation, and inadequate water supply. Most of the drinking water comes from rivers after treatment by chlorination method, and from wells without treatment directly to the houses through the pipeline and is consumed in its natural form. In 2015, diarrheal and gastroenteritis were the fifth leading cause of hospital admission in Khartoum (SASRs 2015). Other studies that investigated the viral and bacterial etiology and related clinical and epidemiological factors in children with acute diarrhea in Khartoum State reported that enteroinvasive *E. coli* (EIEC) was the predominantly found bacterial pathogen, followed by enterohemorrhagic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC) (Adam et al. 2018). Therefore, the main aim of this study was to detect the presence and measure the prevalence of the virulence factors, *stx* (EHEC), *eae* (EPEC), *ipaH* (EIEC), and *aggR* (EAEC) of DEC strains in drinking water in Khartoum State. Also, the antimicrobial-resistant profile for these isolated DEC strains was evaluated.

## METHODOLOGY

### Description of study area and collection of water samples

This study, a descriptive cross-sectional study, was conducted to determine the presence and prevalence of the

virulence factors of DEC strains in drinking water in Khartoum State (Sudan) during the period from April to May 2016.

A total of 46 samples (small sample size due to financial constraints) were collected randomly from different provinces in the state (Bahri, Khartoum, and Omdurman). Samples were collected from different places in provinces (houses, dormitory, company, pharmacy, and cafeteria) and different types of drinking water source (tap water, cooler, and tanks) because these are the sources people regularly drink directly from. Fifty mL of water was collected in sterile screw cap bottles containing about 50 mL of lauryl tryptose broth from places suspected of having fecal contamination in different parts of Khartoum State. All collected water samples in this study come from river water after treatment with chlorine.

### Total and thermotolerant coliforms

The cultures were used for the detection of coliform bacteria in water using the presence-absence coliform test (Clark 1980). The bottle content (50 mL) of sterile selective culture broth containing lactose and an indicator (laury tryptose) (lactose) broth was added to an equal volume (50 mL) of the water sample with a Durham tube. After incubation (48 hours at 37 °C), the positive culture (lactose fermentation with acid and gas production) was cultured in two bottles of Brilliant Green Broth (BGBB) media with a Durham tube. One bottle was incubated for 48 hours at 37 °C to detect the coliform and the other bottle at 44 °C for the detection of heat tolerant coliforms (faecal coliform) (Alraheer 2000). Then, the bacteria were purified and identified by Gram stain, culture on EMB media, and biochemical tests (Kligler Iron Agar (KIA), indole, urease, and citrate utilization test).

### Antimicrobial susceptibility test

Antibiotic susceptibility test was done for all isolated *E. coli* by Kirby-Bauer disk diffusion technique (Biemer 1973) for chloramphenicol 30 µg, ceftriaxone 30 µg, ciprofloxacin 5 µg, gentamicin 10 µg, and tetracycline 30 µg, as per the recommendation of Clinical and Laboratory Standards Institute (CLSI). Briefly, bacterial suspension was prepared

by using a sterile loop, four to five colonies of similar appearance were taken and suspended in 2 mL of sterile saline, and the saline tube was vortexed to create a smooth suspension. The suspension was compared with turbidity standard of McFarland 0.5 to adjust the density of the test suspension. A sterile cotton swab was immersed into the inoculum tubes, and the swab was rotated against the side of the tubes using firm pressure to remove excess fluid. The swab was streaked all over the surface of Mueller–Hinton plate three times. Finally, the swab was passed around the edge of the agar surface. Inoculum was left to dry for a few minutes at room temperature until the liquid dried. Sterile forceps were used to place the anti-microbial disk uniformly and slightly pressed. The plates were then incubated at 35 °C overnight. After overnight incubation, the zone size of inhibition was measured and recorded in mm by using a ruler. Zone size was interpreted according to CLSI guidelines (CLSI 2017). The results of the susceptibility test were reported as susceptible, intermediate, and resistant.

### DNA extraction

The DNA was extracted from all isolated *E. coli* by boiling centrifugation method, as described by Al-Gallas *et al.* (2002). Briefly, several colonies of the isolated organism (*E. coli*) were subcultured in nutrient agar media, and after overnight incubation at 37 °C, one to three colonies were washed with 1 mL sterile normal saline (NS) in a sterile 1.5 mL Eppendorf tube. The tube was centrifuged at 10,000 r/min for 1–2 min. The supernatant was discarded. The pellet was re-suspended in 200 µL of distilled water. Then the tube was boiled at 100 °C for 15 min, followed by centrifugation of the lysate at 13,000 r/min for 3 min. A 150 µL sample of the supernatant was stored at –20 °C (in aliquot) as a template DNA stock and used as the target for PCR assays.

### Measurement of DNA concentration

The concentration of extracted DNA was read using gel electrophoresis to show the presence and quality of DNA in the sample when compared with a DNA marker of known concentration.

## Multiplex polymerase chain reaction (MPCR)

Multiplex PCR (Techne Tc.312 Thermal cycle, UK) was used for the detection of target genes, *ipaH* for EIEC, *aggR* for EAEC, *stx* for EHEC, and *eae* for EPEC, by using specific primer pairs as shown in Supplementary material, Table S1 (Ogata *et al.* 2002; Phantouamath *et al.* 2003; Saad *et al.* 2011).

## Preparation of reaction mixture

The following reagents were used for each reaction in volumes of 25  $\mu$ L in 0.2 mL Eppendorf tubes as follows: 17  $\mu$ L distilled water, 5  $\mu$ L master mix (iNtRoN Biotechnology, Korea), 0.5  $\mu$ L forward primer (Macrogen Company, Korea), 0.5  $\mu$ L reverse primer (Macrogen Company, Korea), and 2.0  $\mu$ L DNA (Template DNA). The PCR mixture was subjected to an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 30 s and the final extension at 72 °C for 5 min.

## Visualization of PCR products

Agarose (2%) was used to make an agarose casting tray flooded by 10X TBE buffer near the gel cover surface, then 5  $\mu$ L of amplified PCR products of each sample was put into each well. To the first well of the casting tray 3  $\mu$ L of DNA ladder (100 bp) was added for each run. The gel electrophoresis apparatus was connected to the power supply (rimer, 125 V, 500 MA, UK). The electrophoresis was done at 100 V for 2 h. After that, the gel was removed from the gel holder and visualized by a UV transilluminator (UVP, UK), and the gel results were photographed using a Polaroid film.

## Statistical analysis

Statistical analysis for the prevalence of *E. coli* virulent genes in different water samples was performed using a Microsoft Excel spreadsheet and presented as a percentage or graph.

## RESULTS

### Distribution of isolated organisms

All water samples ( $n = 46$ ) collected from Khartoum State were positive for the presence of coliforms as well as fecal coliforms, thus, 46 *E. coli* bacteria were isolated from drinking water. The distribution of water samples is as follows: 44% (20/46) were from Khartoum province, 35% (16/46) from Omdurman, and 21% (10/46) from Bahri province. Most of the isolates were from tap water 40/46 (87%), 10% (5/46) from water coolers, and 2% (1/46) from tanks, and these 78% (36/46) were from houses and the rest from markets 22% (10/46).

### Antimicrobial susceptibility testing

From 46 isolated *E. coli*, 17% were resistant to chloramphenicol, 30% to ceftriaxone, 24% to ciprofloxacin, and 24% to gentamicin, with a high resistance rate to tetracycline 98% (see Supplementary material, Table 1).

### Genotyping of DEC virulent genes

Out of 46 isolates, 27 (58.6%) were positive for one or two of *ipaH*, *stx*, and *aggR* genes, while the *eae* gene was negative for all isolates. We found 19 (41.4%) isolates negative for all DEC virulent genes used in this study. *ipaH* gene was detected in 10 (12.7%), *aggR* gene detected in 3 (6.5%), and *stx* gene detected in 3 (6.5%). Both *aggR* and *stx* co-existed in 9 (19.5%) and both *aggR* and *ipaH* co-existed in 2 (4.3%) (see Supplementary material, Table S2).

**Table 1** | The antimicrobial susceptibility test of isolated *E. coli*

Antibiotics	Sensitive number (%)	Resistance number (%)	Intermediate number (%)
Chloramphenicol	31 (68%)	8 (17%)	7 (15%)
Ceftriaxone	29 (63%)	4 (9%)	13 (28%)
Ciprofloxacin	24 (52%)	11 (24%)	11 (24%)
Gentamicin	19 (41%)	11 (24%)	16 (35%)
Tetracycline	1 (2%)	45 (98%)	0 (0.0%)

Prevalence of diarrheagenic *E. coli* virulence genes according to the source of water and the place can be seen in Figures 1 and 2.

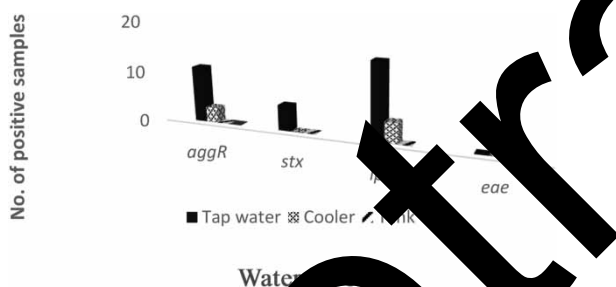
## DISCUSSION

In the present study, we found a high prevalence of EIEC (*ipaH* gene) in the drinking water of Khartoum State, mostly from tap water (32.6%) in houses (30.4%); however, this finding is in disagreement with a study in Japan that reported EPEC was the most common strain in tap water (Yatsuyanagi *et al.* 2003). Also, another study reported that the most common strain was EAEC in the tank water (Dobrowsky *et al.* 2014). This finding may indicate that the tap water was provided with inefficient chlorination. According to WHO guidelines, *E. coli* must not be

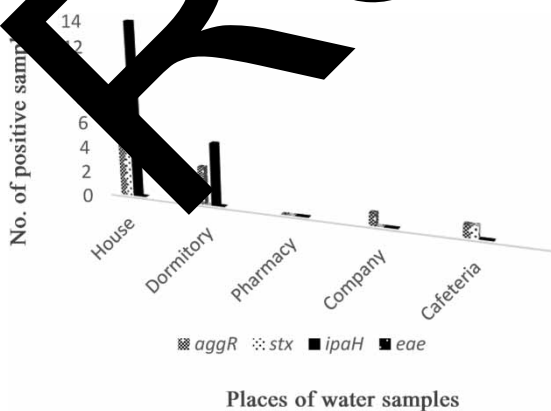
detectable in any 100 mL of drinking water sample (WHO 2011). A commonly used risk classification is based on the number of indicator organisms in a 100-mL sample, which includes: <1, 'very low risk'; 1–5, 'low risk'; 10–100, 'medium risk'; >100, 'high risk' or 'very high risk' (Bain *et al.* 2014). Also, this study found that the prevalence of DEC is higher in Khartoum province (43.5%) and lower in Bahri province (21.7%). This result may be due to the facts that Khartoum province is bigger than Bahri province and consists of many wells, and Khartoum has an old sanitation system with multiple broken pipelines. There was no previous study from Sudan to compare the results with, so this is the first study in the country to report this result.

The antimicrobial sensitivity profile of *E. coli* revealed a high rate of antibiotic resistance to tetracycline (97.8%) and similar findings to this study have been reported in South Africa (defisoye & Okoh 2016). This resistance may be due to long-term usage of tetracycline (introduced in 1950). However, *E. coli* was highly susceptible to chloramphenicol (67.4%), ceftriaxone (63%), ciprofloxacin (57.6%), and gentamicin (41.3%). This finding was different from another study in Europe which reported that antimicrobial resistance to ciprofloxacin, gentamicin, and ceftriaxone is on the rise in European countries (Allocati *et al.* 2013). Another study reported that *E. coli* was resistant to all the antibiotics above (Adugna *et al.* 2015). In Khartoum State, some studies have investigated the sensitivity profile of *E. coli* as the causative agent for urinary tract infection, and reported that most *E. coli* were resistant to ciprofloxacin, gentamicin, tetracycline, chloramphenicol, and ceftriaxone (Hamdan *et al.* 2015; Saeed *et al.* 2017). This indicated the antibiotic resistance by *E. coli* strains that has increased in recent years could mainly be due to high consumption of antibiotics, irrational use, incomplete course of therapy, and use of treatment without prescription from physicians.

The prevalence of virulence factors (*ipaH*, *aggR*, *stx*, and *eae*) associated with EIEC, EAEC, EHEC, and EPEC, respectively, was tested using PCR. It was found that the most prevalent gene detected during this study was *ipaH* (EIEC) ( $N = 10/46$ ) (12.7%), followed by *aggR* (EAEC) and *stx* (EHEC) ( $N = 3/46$ ) (6.5%). This finding might explain the causative agent of acute diarrhea among children in Khartoum State, where EIEC was the predominantly



**Figure 1** | The incidence of virulence genes from *E. coli* isolates according to the type of drinking water source.



**Figure 2** | The presence of virulence genes of *E. coli* isolates according to the place.



detected bacterial pathogen, followed by EHEC and EPEC (Adam *et al.* 2018). This finding is unlike a previous study which reported that the most common pathogen isolated was EPEC followed by EAEC (Canizalez-Roman *et al.* 2013), while others reported that EHEC and EPEC types were the most prevalent (Sidhu *et al.* 2013). Furthermore, Gutiérrez-Jiménez *et al.* (2014) reported that ETEC was the most common, indicating that the water purification system, hygiene practices, and source of water might differ from region to region and from country to country.

Moreover, the results showed a two-combination *E. coli* strain isolated from two samples: *aggR* (EAEC), *stx* (EHEC) ( $N=2/46$ ) (4.3%). This finding was entirely in agreement with a study reporting that 4% of isolates is observed to have a combination of genes from both EHEC and EAEC path types (Sidhu *et al.* 2013), and another study found one sample (0.5%) that contained *stx1* (EHEC) and *aggR* (EAEC) (Trung *et al.* 2016). This finding may reflect recent fresh fecal contamination of surface water with diarrheagenic *E. coli*.

Interestingly, this was the first study to report a combination between the strains of *E. coli* isolated from nine samples that were *aggR* (EAEC) and *ipaH* (EIEC) ( $N=9/46$ ) (19.5%), which was not found in any other previous studies.

The detection of the *eae* gene which encodes intimin which is essential for the intimate attachment of the EPEC to the enterocytes was not found in any drinking water sample. The EPEC pathotype can be classified into two groups, typical EPEC (tEPEC) and atypical EPEC (aEPEC). Humans are the only reservoir for tEPEC, which is transmitted by intimate human contact (Baylis *et al.* 2011). All previous studies that detected this gene were from food samples (Canizalez-Roman *et al.* 2013) and surface water (Sidhu *et al.* 2013). This negative result may be due to all the isolates were from drinking water which might not be contaminated by intimate human contact.

The prevalence of diarrheagenic *E. coli* strains in drinking water in the general population of African countries is reported being as follows: (48%) EAEC in Libya (Ali *et al.* 2012), (35.3%) EPEC in Egypt (Hassanain *et al.* 2015), and (34.7%) EPEC in Chad (Bessimbaye *et al.* 2013). These results differ from the present study findings. This might be due to using a different method for treating drinking water

and to the crucial regional difference in the prevalence of categories of DEC.

## CONCLUSIONS

There is a high prevalence of EIEC (*ipaH* gene) in drinking water in Khartoum State, and there is a high percentage of DEC in tap water, and this may indicate that the contamination of drinking water can be from the source. There is a high percentage of drug resistance between isolated bacteria, thus, the water can also be a source of transmission of drug-resistant bacteria. We recommend that drinking water must be treated and periodically tested for the presence of bacterial contamination, and, also, houses should use filters in tap stands.

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## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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