Shiga Toxigenic *Escherichia* coli in Diarrhetic Pediatric Patients; Virulence factors and Antimicrobial Resistance

Saad Fawwaz Mukhlif,* Amal Al-Hamami,* Maysaa Ibrahim,*

ABSTRACT

Objective: *Escherichia coli* is considered one of the prevalent causes of diarrhea in pediatrics. The current study aimed to assess the diarrhetic and non-diarrhetic *E. coli* virulence markers and antibiotic resistance distribution in pediatrics.

Materials and Methods: Two hundred and sixty stool specimens of diarrhetic and non-diarrhetic pediatrics were collected. The microbial culture was used for *E. coli* isolation and identification. Disk diffusion was applied to assess the *E. coli* isolates antibiotic resistance. PCR appraised the virulence factors distribution.

Results: The mean age of the examined diarrhetic and non-diarrhetic pediatrics were 1.1±0.3 and 1.0±0.4 years, with a 71/49 and 75/65 male to female ratio, respectively. Diarrhea (100%), fever (91.66%), and nausea (66.66%) were frequent symptoms in diarrheal patients. Bloody diarrhea was found in 16.566% of pediatrics. *E. coli* prevalence amongst the diarrhetic and non-diarrhetic pediatric patients was 35% and 7.14%, respectively ($P < 0.05$). The maximum resistance rate was obtained for gentamicin (82.69%), tetracycline (82.69%), ampicillin (80.76%), and penicillin (75%). *E. coli* isolates of pediatric patients presented a higher resistance rate toward all examined antibiotic agents ($P < 0.05$). *Stx1* (44.23%) and *eaeA* (40.38%) were more frequent amongst isolates. *E. coli* isolates of non-diarrhetic pediatric patients only harbored *stx1* (10%), *eaeA* (10%), and *ehlyA* (10%) genes. In total, 3.84% of *E. coli* strains of diarrhetic pediatric patients simultaneously harbored *stx1*, *eaeA*, and *ehlyA* virulence genes. *E. coli* isolates of diarrhetic pediatric patients harboured the highest distribution of all examined virulence genes ($P < 0.05$).

Conclusion and Recommendation: The simultaneous virulence factors and antibiotic resistance distribution in E. coli isolates showed high pathogenicity. Imipenem and cefixime prescription showed effective results against *E. coli* bacteria.

Keywords: Shiga toxigenic *Escherichia coli*, Diarrhea, Pediatrics, Antibiotic resistance, Virulence genes

INTRODUCTION

Escherichia coli (*E. coli*) is a fundamental reason for diarrhea globally[1,2]. Diseases caused by this bacterium are known as bloody and non-bloody diarrhea, fever, nausea and vomiting, weakness, abdominal cramps, and tenesmus[3]. Infants and children are the most vulnerable group to diarrhetic *E. coli*[4].

Diarrheagenic *E. coli* strains are divided into several groups[5]. Enterohemorrhagic *E. coli* (EHEC), a critical subtype of Shiga toxigenic *E. coli* (STEC), is a putative strain that produces Shiga toxin type 1 (STX1, encoded by *stx1* gene) and 2 (STX2, encoded by *stx2* gene), hemolysin (*hlyA*) and intimin (*eaeA*)[6]. These strains are causative of Thrombotic Thrombocytopenic Purpura (TTP), diarrhea, Hemolytic Uremic Syndrome (HUS), and Hemorrhagic Colitis (HC)[7].

STEC-related diseases are chiefly resistant to frequently used antibiotic agents[8]. The literature revealed that STEC bacteria harbored high resistance rates toward different antimicrobial classes, including fluoroquinolone, aminoglycosides, tetracyclines, macrolides, penicillins, cephalosporins, sulfonamides, and glycopeptides[9,10]. Antibiotic-resistant bacteria caused more severe diseases with a longer period with an increment financial load owing to the hospital stay and treatment costs[11,12].

Diarrhea among children is a health concern, and the current study aims to measure the occurrence, antibiotic resistance, and virulence of STEC bacteria isolated from diarrhetic and non-diarrhetic pediatric patients.

MATERIALS AND METHODS

Ethics : Printed consent was taken from the volunteer parents. Personal information of all pediatrics is kept secret. This study was also permitted by the Moral assembly of the Al-Iraqia University, College of Medicine (No. FM/SA/72).

Sampling: From May 2020 to October 2021, 260 stool specimens were collected from pediatric, diarrhetic (120 specimens), and non-diarrhetic (140 specimens). All pediatric patients with diarrhea were comprised in the research. Pediatrics who had a history of antibiotic therapy were excluded from the study. Age, sex, and clinical signs of pediatrics were recorded. Sterile rectal swab specimens were used for stool sampling. Swabs were transferred to the laboratory in tubes containing Stuart medium (Merck, Germany). Transferring was done as soon as possible (within 2 hours) at 4°C.

***E. coli* isolation and identification**

Phosphate buffered saline (PBS, Merck, Germany) was used for sample dilution. First, MacConkey's agar (MC, Merck, Germany) was

* Department of Pediatrics
College of Medicine
Al-iraqia University, Baghdad, Iraq.
E-mail: Saad.f.mukhlif@aliraqia.edu.iq

used to screen the lactose-positive colonies by sample inoculation and incubation at 37°C for 24 h. A typical lactose-positive colony was streaked onto Eosin Methylene Blue (EMB, Merck, Germany) medium and incubated at 37°C for 24 h. Brass green colonies were measured *for E. coli* and identified using biochemical tests[13,14].

Antibiotic susceptibility testing

The *E. coli* antibiotic resistance was evaluated by Kirby–Bauer Mueller–Hinton agar (Merck, Germany)-based disk diffusion in[15,16]. All tests followed the Clinical and Laboratory Standards Institute (CLSI)[17]. The resistance rate of *E. coli* isolates was tested against penicillin (10 µg), gentamicin (10 µg), tetracycline (30 µg), amikacin (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), trimethoprim-sulfamethoxazole (25 µg), cefixime (5 µg), and imipenem (10 µg) (Oxoid, UK). CLSI guidelines were applied for interpretation[18].

Virulence factors PCR detection

DNA was extracted from enriched colonies on the nutrient broth media (Merck, Germany) incubated at 37 °C for 48 h. DNA extraction kit (CinnaGen Co, Iran) was used for DNA extraction from bacterial colonies. All stages were performed, rendering the company's guidelines[19,20]. DNA quality and purity were assessed using previous methods[20-25].

Table 1 shows the criteria used for virulence factors detection using PCR[26]. PCR thermocycler device (Eppendorf, Germany) was used. As positive and negative controls, *E. coli* ATCC 25922 and PCR-grade water (Thermo Fisher Scientific, Germany) were used. Next, electrophoresis and UV analysis were done[27-29].

Statistical analysis

Data were scrutinized by means of the SPSS software rendering Fisher's exact and chi-square tests. A statistical level was determined as P-value < 0.05[30,31].

RESULTS

Demographic characters

Table 2 expresses the populational characters of the examined population. Diarrhetic, and non-diarrhetic pediatrics mean age was 1.1±0.3 and 1.0±0.4 years, with a 71/49 and 75/65 male to female ratio, respectively. None of the non-diarrhetic pediatrics did show diarrhea or bloody diarrhea. Distribution of nausea, diarrhea, bloody diarrhea, fever, abdominal cramps, and tenesmus clinical signs amongst the diarrhetic pediatrics were 66.66%, 100%, 16.66%, 91.66%, 79.16%, and 55%, respectively.

E. coli prevalence

Table 3 expresses *E. coli* incidence amongst the diarrhetic and non-diarrhetic pediatric patients. Fifty- two out of 260 (20%) stool specimens were *E. coli*-positive. *E. coli* frequency amongst the diarrhetic and non-diarrhetic pediatric patients was 35% and 7.14%, respectively ($P < 0.05$).

Antibiotic resistance

Table 4 expresses the *E. coli* antibiotic resistance. Resistance rates toward gentamicin (82.69%), tetracycline (82.69%), ampicillin (80.76%), and penicillin (75%) were higher than others. *E. coli* isolates of diarrhetic pediatric patients harbored the highest resistance rate against inspected antibiotic agents ($P < 0.05$). *E. coli* isolates of diarrhetic pediatric patients harbored the uppermost resistance rate toward gentamicin (90.47%), ampicillin (90.47%), tetracycline (88.09%), and penicillin (83.33%). The lowest resistance rate of *E. coli* isolates of diarrhetic pediatric patients was observed against imipenem (11.90%) and cefixime (23.80%).

Virulence factors distribution

Table 5 expresses the *E. coli* virulence markers distribution. *Stx1* (44.23%) and *eaeA* (40.38%) were more prevalent than others. *Stx2* and *ehlyA* were obtained in 15.38% and 34.61%, respectively. The arithmetically crucial alteration was obtained amid the *stx1* and *stx2* distribution ($P < 0.05$). *E. coli* isolates of diarrhetic pediatric patients harbored the highest distribution of all examined virulence genes ($P < 0.05$). *E. coli* isolates of non-diarrhetic pediatric patients only harbored *stx1* (10%), *eaeA* (10%), and *ehlyA* (10%) genes. *E. coli* bacteria of non-diarrhetic origin did not harbor the combined presence of markers. 3.84% of *E. coli* bacteria of diarrhetic pediatric patients simultaneously harbored *eaeA*, *stx1*, and *ehlyA* virulence genes.

DISCUSSION

The current study assessed the *E. coli* incidence, virulence markers, and antibiotic resistance in diarrhetic and non-diarrhetic pediatric patients. Findings presented a boost *E. coli* bacteria prevalence in diarrhetic specimens. Additionally, *E. coli* bacteria with diarrhetic origin had a higher rate of resistance and virulence markers. This part of our study was comparable to that of Momtaz et al. (2013)[13]. No arithmetical important variance was gotten for the age and sex of examined pediatric patients. However, obtained clinical signs (nausea, diarrhea, bloody diarrhea, fever, abdominal cramps, and tenesmus) were more frequent amongst pediatric patients with diarrhea ($P < 0.05$). Similar clinical signs of *E. coli* diarrheal infections were reported from China[32], Italy[33], and United States[34].

We showed that the *E. coli* incidence amongst the diarrhetic and non-diarrhetic pediatric patients was 35% and 7.14%, respectively. The incidence of *E. coli* in diarrhetic pediatric patients examined in Romania[35], Brazil[36], Sweden[37], Iran[38], and Ethiopia[39] was 6.40%, 45.20%, 1.84%, 75%, and 15.30%, respectively. In a study conducted by Momtaz et al. (2013)[13], *E. coli* incidence amongst the diarrhetic and non-diarrhetic pediatric patients was 68.75% and 36.90%, respectively, which were higher than our records. A study from South Africa[40] revealed that 88.30% of diarrhetic specimens were positive for *E. coli*. They showed that the frequency of DAEC (adhering *E. coli*), EHEC, EPEC (enteropathogenic *E. coli*), and EIEC (enteroinvasive *E. coli*) bacteria amongst the bacteria were 41%, 17%, 17%, and 10%, respectively. As isolates harbored Shiga toxins in our study, they were considered STEC bacteria. Additionally, those *E. coli* isolates that harbored *stx1*, *eaeA*, and *ehlyA* genes together were considered EHEC bacteria (3.84%). In keeping with this, Saka et al. (2019)[41] reported that none of the diarrhetic E. *coli* bacteria of children in Nigeria were related to STEC and EHEC subtypes. In Mexico[42], 23.30% of diarrhetic specimens were positive for *E. coli*. This research indicated that the STEC bacteria were detected in 0.3% of isolates, and there were no positive results for the EHEC subtype. Detected *E. coli* in non-diarrhetic pediatric patients may be related to previous gastrointestinal diseases or microflora because the detected *E. coli* in these patients did not have all virulence factors and harbored a lower incidence of resistance toward antibiotic agents.

E. coli isolates of the pediatric patients of this study harbored a high resistance rate toward some commonly used antimicrobials, especially gentamicin, ampicillin, tetracycline, and penicillin. Over-prescribing

Table 1: Virulence factors molecular detection conditions[26]

Target genes	Primer sequence (5'-3')	PCR product (bp)	PCR programs	PCR volume (50µL)
Stx1	F: AAA TCG CCA TTC GTT GAC TAC TTC T R: TGC CAT TCT GGC AAC TCG CGA TGC A	366	**1 cycle:** 3 min: 95 ^{0C}	10X PCR buffer: 5 µL $Mgcl_{2:}$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL
Stx2	F: CGA TCG TCA CTC ACT GGT TTC ATC A R: GGA TAT TCT CCC CAC TCT GAC ACC	282	**34 cycles:** 60 s: 94 ^{0C}	
eaeA	F: TGC GGC ACA ACA GGC GGC GA R: CGG TCG CCG CAC CAG GAT TC	629	45 s: 56 ^{0C} 60 s: 72 ^{0C}	
ehly	F: CAA TGC AGA TGC AGA TAC CG R: CAG AGA TGT CGT TGC AGC AG	432	**1 cycle:** 10 min: 72 ^{0C}	

Table 2: Populational characters of the studied population

Demographic characters	Individuals (260 people)		*P* value
	Diarrhetic (120 cases)	Non-diarrhetic (140 cases)	
Mean age (SD)	1.1 (0.3)	1.0 (0.4)	0.75
Sex (M/F)	71/49	75/65	0.55
Clinical signs (%)			
Nausea	80 (66.66)	5 (3.57)	0.017
Diarrhea	120 (100)	-	-
Bloody diarrhea	20 (16.66)	-	-
Fever	110 (91.66)	15 (10.71)	0.024
Abdominal cramps	95 (79.16)	4 (2.85)	0.022
Tenesmus	66 (55)	2 (1.42)	0.019

Table 3: *E. coli* incidence amongst the diarrhetic and non-diarrhetic pediatric patients

Pediatrics specimens	N. collected	N (%) positive for *E. coli*
Stool from diarrhetic	120	42 (35)
Stool from non-diarrhetic	140	10 (7.14)
Total	260	52 (20)

Table 4: *E. coli* antibiotic resistance rate

Pediatric stool specimens (N. positive for *E. coli*)	Pattern of resistance against antibiotics (%)								
	P10*	G10	AK30	T30	Tri-Sul	CIP	A10	Cef5	IMP
Diarrhetic (42)	35 (83.33)	38 (90.47)	20 (47.61)	37 (88.09)	17 (40.47)	15 (35.71)	38 (90.47)	10 (23.80)	5 (11.90)
Non-diarrhetic (10)	4 (40)	5 (50)	3 (30)	6 (60)	2 (20)	1 (10)	4 (40)	-	-
Total (52)	39 (75)	43 (82.69)	23 (44.23)	43 (82.69)	19 (36.53)	16 (30.76)	42 (80.76)	10 (19.23)	5 (9.61)

*P10: penicillin (10 µg/disk), G10: gentamicin (10 µg/disk), AK30: amikacin (30 µg/disk), T30: tetracycline (30 µg/disk), Tri-Sul: trimethoprim-sulfamethoxazole (25 µg/disk), CIP: ciprofloxacin (5 µg/disk), A10: ampicillin (10 µg/disk), Cef5: cefixime (5 µg/disk), IMP: imipenem (10 µg/disk).

Table 5: *E. coli* virulence markers distribution

Pediatric stool specimens (N. positive for *E. coli*)	Virulence factors distribution (%)									
	stx1	*stx2*	*eaeA*	*ehlyA*	*stx1+stx2*	*stx1+eaeA*	*stx1+ehlyA*	*stx2+eaeA*	*stx2+ehlyA*	*stx1+eaeA+ehlyA*
Diarrhetic (42)	22 (52.38)	8 (19.04)	20 (47.61)	17 (40.47)	5 (11.90)	13 (30.95)	14 (33.33)	4 (9.52)	5 (11.90)	2 (4.76)
Non-diarrhetic (10)	1 (10)	-	1 (10)	1 (10)	-	-	-	-	-	-
Total (52)	23 (44.23)	8 (15.38)	21 (40.38)	18 (34.61)	5 (9.61)	13 (25)	14 (26.92)	4 (7.69)	5 (9.61)	2 (3.84)

antibiotics and their usage without any attention to the time interval, excessive use of disinfectants, and selling over-the-counter antibiotics are among the possible details for the boost rate of antibiotic resistance. In Iraq[43], *E. coli* isolates of diarrhetic children showed complete resistance toward cefodizime, carbenicillin, piperacillin, and imipenem. In Egypt[44] diarrhetic *E. coli* bacteria recovered from pediatric patients harbored the uppermost resistance rate against trimethoprim-sulfamethoxazole (62.10%), amikacin (24.20%), cefoxitin (22.70%), ceftriaxone (66.60%), meropenem (9.10%), tetracycline (77.30%), ceftazidime (72.70%), and amoxicillin-clavulanate (60.60%). Similarly, in Ethiopia[45], diarrhetic *E. coli* bacteria isolated from pediatric patients harbored the uppermost resistance rate against ampicillin (83.60%) and augmentin (83.60%), trimethoprim-sulfamethoxazole (62.30%), chloramphenicol (21.30%), and nalidixic acid (19.70%). Similar to our findings, the high resistance rate of diarrhetic *E. coli* against gentamicin, ampicillin, tetracycline, and penicillin antimicrobials was reported by Omolajaiye et al. (2020)[46] (South Africa), Cho et al. (2011)[47] (Korea), and Uddin et al. (2021)[48] (Bangladesh). We found the high susceptibility of *E. coli* bacteria against imipenem. This part of our study was similar to previous studies[49,50]. Jafari et al. (2021)[51] showed that diarrhetic *E. coli* bacteria displayed a high susceptibility against imipenem (99.40%), piperacillin (96%), and amikacin (93.10%). Imipenem is a carbapenem family member, a clinically significant antimicrobial family used to treat Multidrug-Resistant (MDR) bacterial infections. Thus, it is not astonishing that *E. coli* bacteria showed low resistance to this agent.

E. coli isolates harbored several virulence factors, largely *stx1* and *eaeA*. Some isolates harbored several virulence factors simultaneously. This matter may show the *E. coli* bacteria high pathogenicity. Some isolates of healthy pediatrics also harbored these genes, but only *stx1*, *eaeA*, and *ehlyA* were detected in 10% of isolates. Virulent isolates of healthy pediatrics appear to have originated from previous infections or developing ones in the previous days. The simultaneous presence of *stx2*, *stx1*, *ehlyA*, and *eaeA* virulence markers in the *E. coli* bacteria isolated from clinical specimens have been reported previously[52,53]. However, none of the *E. coli* isolates of a research conducted by Sirous et al. (2020)[54] did not harbor *stx* and *eae* genes, which showed the absence of STEC bacteria. Put together, using novel techniques in medical sciences can diminish the risk of diseases[55-57].

CONCLUSION AND RECOMMENDATIONS

The current research showed the high incidence of virulent and antibiotic-resistant *E. coli* bacteria amongst diarrhetic pediatric patients. Some isolates were resistant to some tested antimicrobials, and others harbored two or three virulence factors simultaneously. This matter may show the *E. coli* bacteria boost pathogenicity of in diarrhetic pediatric patients. According to antibiogram testing, imipenem and cefixime prescription may be helpful for the treatment of diarrheal cases in pediatrics. Our findings showed that most bacteria were related to STEC subtypes as they harbored *eaeA*, *stx2*, *stx1*, and *ehlyA* genes. Some bacteria harbored *eaeA*, *stx1*, and *ehlyA* genes together, which may show that they are related to EHEC subtypes. However, additional studies should identify the meticulous character of STEC and EHEC bacteria in pediatric diarrheal specimens.

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Potential Conflict of Interest: None

Competing Interest: None

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