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Shiga Toxigenic Escherichia Coli Antimicrobial Resistance Properties in Diabetic and Nondiabetic Pediatric Patients; A Case-Control Study

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Abstract

Background

Resistant Shiga toxigenic Escherichia coli (STEC), is the most prevalent source of diarrhea in pediatrics. This study was conducted to investigate the antimicrobial resistance properties of STEC strains of diabetic and non-diabetic pediatrics with diarrhea.

Materials and Methods: This was a case-control study conducted from December 2014 to September 2015 in an educational hospital, Jiroft city, Iran. Diarrheic stool samples were collected from diabetic (n= 385) and non-diabetic (n= 300) pediatrics. The samples were cultured and the STEC strains were tested by disk diffusion and polymerase chain reaction (PCR) amplification were applied for detecting antibiotic resistance genes.

Results

Sampling was performed from 685 patients (51.8% male). Total prevalence of STEC strains in diabetic and non-diabetic pediatrics were 6.5% and 3.0%, respectively (P = 0.007). Prevalence of the gens that encode resistance against ampicillin (CITM), fluoroquinolone (qnr), trimethoprim (dfrA1), tetracycline (tetA), gentamicin [aac(3)-IV] and sulfonamide (sul1) were 97.1%, 64.7%, 61.8%, 58.8%, 58.3% and 52.9%, respectively. Non-diabetic pediatrics harbored the lower prevalence of antibiotic resistance genes (P = 0.034).

Conclusion

High numbers of STEC, especially O157 strains, showed a multidrug-resistance against ampicillin, ciprofloxacin, gentamycin, sulfamethoxazole, and tetracycline. *CITM*, *qnr*, *dfrA1*, *tetA*, [*aac(3)-IV]* and *sul1* antibiotic resistance genes were identified in the STEC strains of diarrheic samples of diabetic and non-diabetic pediatric patients.

Key Words: Antimicrobial resistance properties, Diabetes, Diarrhea, Pediatrics, Shiga toxin producing Escherichia coli.

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1- INTRODUCTION

Diabetes is among the most prevalent non-infectious diseases in pediatrics (1, 2). Pediatrics less than 10 years of age are more commonly affected by type 1 diabetes and is the leading cause of diabetes in pediatrics of all ages. Type 1 diabetes is an autoimmune disease that destroyed insulin-producing beta-cells of the pancreas (1-4). It was assumed that immunity level have been reduced in pediatrics suffered from diabetes (5, 6). Therefore, they may vulnerable to several types of infections. Pediatrics are in close contact with the polluted environment. They always use from the foods which are supply in outside. Due to the low levels of hygiene and public health in the restaurant and also food handlers outside the home, the possibilities of occurrence of gastrointestinal infections is undeniable. One of the most common types of gastrointestinal disorders is diarrhea (7-9).

Diarrhea is usually resulted from gastrointestinal infections caused by various types of bacteria, viruses, or parasites. The causative microorganism that induced diarrhea can vary based on geographic region, level of hygiene and economic situation of the community. However, epidemiological investigations revealed that Escherichia coli (E. coli) is one of the most prevalent cause of diarrhea in pediatrics (7, 8). E. coli is a gram negative bacilli from Enterobacteriaceae family and is typically classified into enteroinvasive E. coli (EIEC), enterotoxigenic E. coli (ETEC), enteroadherent E. coli (EAEC), enteropathogenic E. coli (EPEC), enterohemorrhagic E. coli (EHEC), and diffusely adherent E. coli (DAEC) subgroups (10). EHEC strains are a subdivision of Shiga-toxigenic E. coli (STEC) strains. STEC strains are responsible for severe clinical symptoms such as uncomplicated diarrhea, bloody diarrhea, hemorrhagic colitis (HC), thrombocytopenia, hemolytic anemia, lethal hemolytic uremic syndrome (HUS) and acute renal failure (11). Unfortunately, therapeutic options have significantly reduced due to the occurrence of multi drug resistant strains of this bacterium (12-15). It seems that, antibiotic resistant STEC may lead to more severe diseases (12-16). Antibiotic resistance in STEC strains may accompanied with the presences of some antibiotic resistance genes in the nucleotide of these Bactria (12-16). The genes that encode resistance against tetracycline (*tetA* and *tetB*), trimethoprim (*dfrA1*), aminoglycosides (*aadA1*), fluoroquinolone (*qnr*), gentamicin [*aac(3)-IV]*, sulfonamide (*sul1*), cephalothin (*blaSHV*), ampicillin (*CITM*), erythromycin (*ereA*) and chloramphenicol (*cat1* and *cmlA*) were the most commonly antibiotic resistance genes detected in the resistant STEC strains (12-16). Imperative information about distribution of antimicrobial resistance properties in STEC strains isolated from diabetic pediatric patients suffered from diarrhea are limited in the world. Therefore, this study was conducted in order to investigate the antimicrobial resistance properties of STEC strains isolated from Iranian diabetic and non-diabetic pediatric patients suffered from diarrhea.

2- MATERIALS AND METHODS

2-1. Study design and ethical consideration

This was a case-control study conducted from December 2014 to September 2015 in an educational hospital, Jiroft, Iran. The protocol of the present study was approved by the ethics committee of the Jiroft University of Medical Sciences (ETH 10552). This study did not entrap the patients' medical care and did not cause any extra cost for the subjects. The aim and advantages of the research explained, and all samples were taken from volunteer

patients. The investigators were adhered to declaration of Helsinki principles throughout the study.

2-2. Patients

Consecutive sampling method was considered in this study. All pediatrics admitted in emergency department with chief complaint of diarrhea, were enrolled. Those who were known as diabetic type 1 or had blood sugar level > 200 mg/dl allocated to case group; and the others were subjected to control group. Sampling continued until the calculated sample size was determined. Diabetic and non-diabetic pediatric patients were classified into six groups based on their age (< 2 years, 2-4 years, 4-6 years, 6-8 years and > 8 years old). Information about the clinical and epidemiological history of patients, were obtained using a pre-prepared checklist. Sample size was calculated using $n = z^2 \frac{pq}{d^2}$ formulae, and eventually 685 diarrheic stool samples from diabetic (n = 385) and non-diabetic (n = 300) pediatrics were collected. Stool samples were collected using sterile rectal swabs. All swabs were placed into tubes containing Stuart medium. Samples were transferred to the laboratory at 4°C in a cooler with iced-packs.

2-3. Isolation of Shiga toxigenic Escherichia coli

Ten mL of each sample was mixed with 90 mL trypton soya broth (Oxoid) supplemented with novobiocin (20 mg/L, Sigma, Germany). After homogenization process, the samples were incubated at the temperature of 37°C for 18-24 hours. A total 100 µl of cultures were plated on Sorbitol MacConkey Agar (Oxoid) plates containing Cefixime-tellurite Supplement (Oxoid). The samples were incubated at the temperature of 42°C for 24 hours in this step. Then sorbitol negative colonies were tested for the presence of O157 antigen by latex agglutination (Oxoid).

2-4. Antimicrobial susceptibility testing

According to the clinical and laboratory standards institute (CLSI) guidelines, using Mueller–Hinton agar (Merck, Germany), antimicrobial susceptibility test was performed by the Kirby–Bauer disc diffusion method (17). After incubating the inoculated plate in an aerobic atmosphere for 18-24 hours at 37 °C, the susceptibility of the isolated E. coli to the was assessed. Antimicrobial agents include: ampicillin (10 u/disk); cephalothin (30 µg/disk); chloramphenicol (30 µg/disk); ciprofloxacin (5 µg/disk); enrofloxacin (5 µg/disk); gentamycin (10 µg/disk); nitrofurantoin (300 µg/disk); penicillin (10 u/disk); streptomycin (10 µg/disk); sulfamethoxazole (25 µg/disk); sulfonamides (100 µg/disk); tetracycline (30 µg/disk); trimethoprim (5 µg/disk).
The results were interpreted in accordance with interpretive criteria provided by CLSI. E. coli ATCC 25922 was used as quality control organisms in antimicrobial susceptibility determination.

2-5. DNA extraction

Bacterial strains were overnight grown at 37ºC, in Trypticase Soy Agar (TSA, Merck, Germany). One colony was suspended in 100 µL of sterile distilled water. Boiling the suspension for 13 minutes was followed by freezing and subsequently centrifuged at 14000 rpm for 15 minutes to pellet the cell debris. The supernatant was used as a template for amplification reaction.

2-6. Amplification of antibiotic resistance genes

To detect antibiotic resistance genes of STEC isolates, a polymerase chain reaction (PCR) assays was used. The primer sequences and PCR programs (temperatures and volumes) used for amplification of STEC strains are summarized in **Table.1**. PCR program and volumes of reaction are shown in **Table.2**. All the PCR reactions were performed in

thermocycler (Mastercycler gradient Eppendorf, Germany), and PCR products were visualized by electrophoresis in 1.5% agarose gel, strained with ethidium bromide, and examined under ultraviolet illumination. Strains of E. coli O157:K88ac:H19, CAPM 5933 and E. coli O159:H20, CAPM 6006 were used as positive controls and distilled water was used as negative control (**Figure.1**).

2-7. Statistical analysis

The data were analyzed using IBM© SPSS© Statistics version 21.0 (IBM© Corp., Armonk, NY, USA) and p-value was calculated using Chi-square and Fisher's exact tests to find any significant relationship between various ages, sexes and antibiotic resistance properties of STEC strains isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea. The p-value less than 0.05 was considered statistically significant.

Table-1: Primers used for detection of antimicrobial resistant genes in Shiga toxin-producing *Escherichia coli* isolated from diabetic and non-diabetic pediatric patients

Target gene	Primers name	Primer sequences (5'–3')	Product size (bp)	Reference
aadA1	Streptomycin	(F) TATCCAGCTAAGCGCGAACT (R) ATTTGCCGACTACCTTGGTC	447	(16)
tetA	Tetracycline	(F) GGTTCACTCGAACGACGTCA (R) CTGTCCGACAAGTTGCATGA	577	(16)
tetB	Tetracycline	(F) CCTCAGCTTCTCAACGCGTG (R) GCACCTTGCTGATGACTCTT	634	(16)
dfrA1	Trimethoprim	(F) GGAGTGCCAAAGGTGAACAGC (R) GAGGCGAAGTCTTGGGTAAAAAC	367	(17)
qnr	Fluoroquinolone	(F) GGGTATGGATATTATTGATAAAG (R) CTAATCCGGCAGCACTATTTA	670	(18)
aac(3)-IV	Gentamicin	(F) CTTCAGGATGGCAAGTTGGT (R) TCATCTCGTTCTCCGCTCAT	286	(19)
sul1	Sulfonamide	(F) TTCGGCATTCTGAATCTCAC (R) ATGATCTAACCCTCGGTCTC	822	(19)
blaSHV	Cephalothin	(F) TCGCCTGTGTATTATCTCCC (R) CGCAGATAAATCACCACAATG	768	(19)
CITM	Ampicillin	(F) TGGCCAGAACTGACAGGCAAA (R) TTTCTCCTGAACGTGGCTGGC	462	(19)
cat1	Chloramphenicol	(F) AGTTGCTCAATGTACCTATAACC (R) TTGTAATTCATTAAGCATTCTGCC	547	(19)
cmlA	Chloramphenicol	(F) CCGCCACGGTGTTGTTGTTATC (R) CACCTTGCCTGCCCATCATTAG	698	(19)

Table-2: Polymerase chain reaction (PCR) conditions for detection of antimicrobial resistance genes in Shiga toxin-producing *Escherichia coli* isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea

Genes	PCR program	PCR volume (50 µL)
aadA1, tetA, tetB, dfrA1, qnr, aac(3)-IV, sul1, blaSHV, CITM, cat1, cmlA	1 cycle: 94 ^{0C} ---------- 8 min. 32 cycle: 95 ^{0C} ---------- 60 s 55 ^{0C} ---------- 70 s 72 ^{0C} ---------- 2 min 1 cycle: 72 ^{0C} ---------- 8 min	5 µL PCR buffer 10X 2.5 mM $Mgcl_2$ 200 µM dNTP (Fermentas) 0.5 µM of each primers F & R 2 U Taq DNA polymerase (Fermentas) 3 µL DNA template

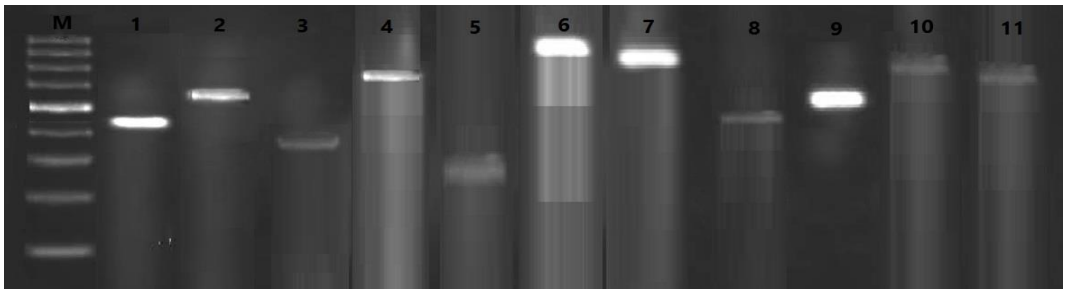

Fig.1: Results of the gel electrophoresis for PCR amplification of antibiotic resistance genes. M: 100 bp ladder, 1-11: Positive samples for various antibiotic resistance genes.

3- RESULTS

Sampling was performed from 685 patients including 355 (51.8%) male and 330 (48.2%) female, and 385 (56.2%) diabetic and 300 (43.8%) non-diabetic patients. There were 202 (29.5%) cases more than 8, 172 (25.1%) cases between 6-8, 143 (20.9%) cases between 4-6, 91 (13.3%) cases between 2 to 4 and 77 (11.2%) cases less than 2 years old.

Table.3 represents the total prevalence of STEC strains in diabetic and non-diabetic pediatric patients suffered from diarrhea. Ten out of 195 (5.1%) samples taken from male diabetic pediatrics, 15 out of 190 (7.89%) samples taken from female diabetic pediatrics, 4 out of 160 (2.5%) samples of male non-diabetic pediatrics, and finally 5 out of 140 (3.6%) samples of female non-diabetic pediatrics were positive for STEC strains. There were no positive results in lower than 2 years old male and female diabetic and non-diabetic pediatrics. Besides, 2-4 years old male pediatrics had no positive results for STEC strains. Statistically significant differences were seen for the prevalence of STEC strains between diabetic and non-diabetic pediatrics ($P = 0.007$), male and female pediatrics ($P = 0.025$), and also between various age groups ($P = 0.018$).

Table.4 represents the antimicrobial resistance pattern of the STEC strains isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea. **Table.4** shows the antimicrobial resistance pattern of the STEC strains isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea. We found that the STEC strains of diabetic pediatrics harbored the highest levels of resistance against ampicillin (100%), gentamycin (94.1%), tetracycline (88.2%), ciprofloxacin (79.4%) and sulfamethoxazole (70.6%). STEC strains of non-diabetic pediatric patients harbored the lower prevalence of antibiotic resistance. Statistically significant difference was seen for the prevalence of antibiotic resistance between diabetic and non-diabetic pediatrics ($P = 0.047$).

Table.5 represents the distribution of antibiotic resistance genes in the STEC strains isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea. Prevalence of the gens that encode resistance against ampicillin (*CITM*), fluoroquinolone (*qnr*), trimethoprim (*dfrA1*), tetracycline (*tetA*), gentamicin (*aac(3)-IV*) and sulfonamide (*sul1*) were 97.1%, 64.7%, 61.8%, 58.8%, 58.3% and 52.9%, respectively. Non-diabetic pediatrics harbored the lower prevalence of antibiotic resistance genes. Statistically significant difference was seen regarding the prevalence of antibiotic resistance genes between diabetic and non-diabetic pediatrics ($P = 0.034$).

Table-3: Distribution of Shiga toxigenic Escherichia coli (STEC) strains in diabetic and non-diabetic pediatric patients suffered from diarrhea

Variables		No. samples	No. STEC (%)
Diabetic			
Male	< 2 years	20	0 (0.00)
	2-4 years	25	1 (4.0)
	4-6 years	40	2 (5.0)
	6-8 years	50	3 (6.0)
	> 8 years	60	4 (6.66)
	Total	195	10 (5.12)
Female	< 2 years	10	0 (0.00)
	2-4 years	17	1 (5.88)
	4-6 years	46	4 (8.69)
	6-8 years	55	5 (9.09)
	> 8 years	62	5 (8.06)
	Total	190	15 (7.89)
Non-diabetic			
Male	< 2 years	25	-
	2-4 years	25	-
	4-6 years	28	1 (3.57)
	6-8 years	37	1 (3.70)
	> 8 years	45	2 (4.44)
	Total	160	4 (2.50)
Female	< 2 years	22	0 (0.00)
	2-4 years	24	1 (4.16)
	4-6 years	29	1 (3.44)
	6-8 years	30	1 (3.33)
	> 8 years	35	2 (5.71)
	Total	140	5 (3.57)

Table-4: Antimicrobial resistance pattern of the STEC strains isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea

Antimicrobial resistance pattern	Groups (No. positive)		Total (n=34)
	Diabetic (n=25)	Non-diabetic (n=9)	
Tetracycline (30 µg/disk)	23 (92)	7 (77.77)	30 (88.23)
Streptomycin (10 µg/disk)	12 (48)	3 (33.33)	15 (44.11)
Chloramphenicol (30 µg/disk)	3 (12)	0 (0.00)	3 (8.82)
Sulfamethoxazole (25 µg/disk)	19 (76)	5 (55.55)	24 (70.58)
Gentamycin (10 µg/disk)	25 (100)	7 (77.77)	32 (94.11)
Enrofloxacin (5 µg/disk)	14 (56)	4 (44.44)	18 (52.94)
Cephalothin (30 µg/disk)	12 (48)	2 (22.22)	14 (41.17)
Ciprofloxacin (5 µg/disk)	23 (92)	4 (44.44)	27 (79.41)
Trimethoprim (5 µg/disk)	20 (80)	3 (33.33)	23 (67.64)
Nitrofurantoin (300 µg/disk)	7 (28)	3 (33.33)	10 (29.41)
Ampicillin (10 u/disk)	25 (100)	9 (100)	34 (100)
Penicillin (10 u/disk)	17 (68)	3 (33.33)	20 (58.82)

Table-5: Distribution of antibiotic resistance genes in the STEC strains isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea

Antibiotic resistance genes	Groups (No. positive)		Total (n=34)
	Diabetic (n=25)	Non-diabetic (n=9)	
aadA1 (streptomycin)	11 (44)	2 (22.22)	13 (38.23)
*tetA (*tetracycline)	17 (68)	3 (33.33)	20 (58.82)
*tetB (*tetracycline)	7 (28)	1 (11.11)	8 (23.52)
*dfrA1 (*trimethoprim)	19 (76)	2 (22.22)	21 (61.76)
*Qnr (*fluoroquinolone)	18 (72)	4 (44.44)	22 (64.70)
*aac(3)-IV (*gentamicin)	25 (100)	4 (44.44)	29 (58.29)
*sul1 (*sulfonamide)	14 (56)	4 (44.44)	18 (52.94)
*blaSHV (*cephalothin)	14 (56)	1 (11.11)	15 (44.11)
*CITM (*ampicillin)	25 (100)	8 (88.88)	33 (97.05)
*cat1 (*chloramphenicol)	2 (8)	0 (0.00)	2 (5.88)
*cmlA (*chloramphenicol)	1 (4)	0 (0.00)	1 (2.94)

4- DISCUSSION

The results of the present study showed that the prevalence of resistant strains of Shiga toxigenic *E. coli* in diabetic diarrheic pediatrics was higher than non-diabetic ones. Total prevalence of STEC strains was 6.49% in current study. High prevalence of diarrhea caused by *E. coli* strains have been reported previously (18, 19). The results of the previous study revealed that survivors with diarrhea-associated HUS caused by *E. c*oli O157:H7 have a significantly increased incidence of diabetes due to complete insulin deficiency, which may relapse years after the primary infection episode. However, less severe forms of infection, such as *E. coli* O157:H7 induced gastroenteritis without HUS, do not increase the risk of type 2 diabetes (19).

Previous investigation which was conducted by Dormanesh et al. in Iran, revealed that of the 480 samples taken from pediatrics suffered from diarrhea and healthy ones, 40.6% samples were positive for *E. coli*. On the other hand, 59% diarrheic stool samples and 27.5% of non-diarrheic stool samples were positive (20). The amount of positive for *E. coli* in Dormanesh study was entirely higher than our investigation. It may be possible to justify that we studied only on STEC strains and especially O157 serogroup of the *E. coli*. O157-*E. coli* is a highly pathogenic bacterium which is associated with foods with animal origin and especially raw meat and milk. Low prevalence of these strains in our study is maybe due to the low ages of pediatric patients. In the other hand, pediatric of these ages cannot use from the raw meat and milk which are the main sources of O157 strains. Therefore, it is not surprising that lower than 2 years and even 2-4 years old pediatrics had the low prevalence of STEC strains. The results of the Mattar et al., was similar to our research.

They revealed that of 300 diarrhea specimens collected from pediatrics, 14 strains corresponded to *E. coli* O157:H7 with a prevalence rate of 4.7% in pediatrics with acute gastroenteritis. The prevalence was 1.14%, the excess of risk of presenting *E. coli* O157:H7 was 14% in pediatrics with acute gastroenteritis. In three of the 85 controls *E. coli* O157:H7 was isolated, with a prevalence rate of 3.53%. The mean age of the 14 patients was 21 months (range: 3 months to 7 years) (21). STEC strains of our investigation harbored the highest levels of resistance against ampicillin, gentamycin,

tetracycline, ciprofloxacin and sulfamethoxazole with respect to high prevalence of *CITM*, *qnr*, *dfrA1*, *tetA*, *aac(3)-IV* and *sul1* antibiotic resistance genes. Dormanesh et al. reported that the most commonly detected antibiotic resistance genes in the STEC strains of diarrheic pediatrics were *CITM* (80.30%), *aac(3)-IV* (75.75%) and *tetA* (65.15%) which was similar to our results (20). In a study which was conducted on Bangladesh, the *E. coli* strains isolated from the cases of diarrhea in pediatrics harbored the high levels of resistance against ampicillin (100%), ceftriaxone (77.41%), nalidixic acid (70.96%), ciprofloxacin (61.29%), cotrimoxazole (45.16%), and tetracycline (41.93%) which was similar to our results (22).

High prevalence of antibiotic resistance in the STEC strains of our study is maybe due to the indiscriminate and irregular prescription of antibiotics by medical and veterinary practitioners. Our results showed that 8.82% of STEC strains were resistance to chloramphenicol. Chloramphenicol is a forbidden antibiotic and the slight antibiotic resistance to this drug in our study indicated the irregular and unauthorized use in medical treatment in Iran. Similar results for resistance against chloramphenicol have been reported previously (23-25). Fazeli and Salehi reported that significant amount of STEC strains isolated from Iranian diarrheal patients were resistant to amoxicillin, tetracycline and trimethoprim-sulfamethoxazole (25). In a study by Mora et al., the highest prevalence of antimicrobial resistance of non-O157 STEC strains of was found against streptomycin, sulfisoxazole, and tetracycline which was in lower rate compare with our results (26). As far as we know, the present study is the first prevalence report of the antimicrobial resistance properties of STEC strains isolated from diabetic and non-diabetic pediatric patients suffered from diarrhea. Diabetic pediatrics harbored the higher levels of infection with STEC strains and antibiotic resistance properties. The prevalence of STEC was higher in diabetic pediatrics versus non-diabetic ones. The most effective antibiotic agents which can use in the cases of diarrhea caused by STEC strains in diabetic patients were nitrofurantoin, streptomycin, and cephalothin.

4-1. Limitations of the study

This study was conducted in only one medical center of a small city, so the prevalence of total community may have influenced on the results. Performing multicenter surveys in various society would be more accurate. Recording more baseline and demographic characteristics beside some other required data, and applying multivariate regression model analysis could assess whether diabetes is a risk factor for occurring antibiotic resistance STEC or not. The current study could not answer this questions.

5- CONCLUSION

High numbers of STEC especially O157 strains, resistance against ampicillin, ciprofloxacin, gentamycin, sulfamethoxazole, and tetracycline. *CITM*, *qnr*, *dfrA1*, *tetA*, *aac(3)-IV* and *sul1* antibiotic resistance genes were identified in the STEC strains of diarrheic samples of diabetic and non-diabetic pediatric patients.

6- AUTHORS' CONTRIBUTION

All authors passed four criteria for authorship contribution based on recommendations of the International Committee of Medical Journal Editors.

7- CONFLICT OF INTEREST: None.

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