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Incidence of Heat-Labile Toxin (LT) Producing Enterotoxigenic *Escherichia coli* Mediated Diarrheal Disease in Egyptian Children under 5 Years Presenting to Tanta University Hospital

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MAAEW and MAS designed the study, carried out the research, performed the statistical analysis and wrote the protocol. Author MAAEW wrote the first draft of the manuscript. Authors MAAEW, MAS and MAN managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Enterotoxigenic *E. coli* (ETEC) is under recognized but an important cause of diarrhea in the developing countries. It is the most frequent bacterial cause of diarrhea in children and adults living in these areas but is most frequently seen in children. We aimed to determine the incidence of LT producing ETEC in cases of acute diarrhea in children under 5 years, to detect the phenotypic characters of LT producing ETEC and to investigate the presence of the heat labile toxigenicity gene in isolated *E. coli* strains.
Methodology: This study was carried out on 100 children with acute diarrhea. After history taking and clinical examination, diarrheal stool was used for phenotypic characterization of ETEC through isolation of *E. coli* by culture followed by microscopic examination then biochemical reactions using API 20 E. LT producing ETEC strains were detected using Phadebact R ETEC-LT test. Antibiotic

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sensitivity of the positive strains was done followed by genotypic characterization through detection of heat-labile toxigenecity gene *(elt)* in isolated *E. coli* strains by polymerase chain reaction.
Results: LT producing ETEC was isolated in (8%) of studied children with a higher sensitivity of PCR than coagglutination test. There was no significant effect on ETEC incidence regarding sex or age; it was significantly lower in breast fed infants with a seasonal peak during summer and early autumn. It showed sensitivity to ceftriaxone (87.5%) and resistance to trimethoprim-sulphamethoxazole (75%), ampicillin (62.5%) and gentamycin (50%).
Conclusion: Rapid screening test is a useful addition to laboratory diagnostic procedures in regions where ETEC is endemic. PCR has high levels of sensitivity and specificity and able of detecting both expressed and silent genes. Sensitivity of isolates to ceftriaxone (87.5%) suggests that this antibiotic is a rational choice for the treatment of prolonged childhood diarrhea in which ETEC is a primary etiologic causative agent.

Keywords: Diarrhea; children; ETEC; coagglutination; PCR.

1. INTRODUCTION

Diarrheal disease is representing one of the major health problems in the developing countries and is considered the major cause of death in children accounting for about two million deaths per year all over the world [1,2].

Many bacterial, viral and parasitic enteropathogens are incriminated as a cause of acute diarrheal syndromes either as a single or multiple etiologies [3].

According to clinical, epidemiological and molecular criteria, *E. coli* associated with diarrhea are classified into enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC) [4,5].

Enterotoxigenic *E. coli* (ETEC) is among the major etiological agents of gastroenteritis followed by *rotavirus*, *Vibrio cholerae*, and *Shigella* spp. leading to an estimated 1.5 million deaths each year all over the world and nearly 1.4 billion diarrhea episodes occurred every year among children under 5 years of age in socioeconomically developing countries [1,6,7]. ETEC was first recognized as a cause of human illness in 1960s [8]. It is also a major cause of traveler's diarrhea and the most common pathogen among the diarrheagenic categories of *E. coli*, especially in the developing world as it always associated with poor hygiene and sanitation [9,10].

ETEC is reported as the second most common cause of diarrhea after rotavirus in pre-school children according to the World Health Organization (WHO), and therefore a vaccine development is urgently needed [11].

Diarrhea due to ETEC usually develop after ingestion of contaminated food or water and initiated within 3 days after ingestion of approximately 10^8 to 10^{10} organisms [12,13]. The disease varies from a mild illness to one of great severity, usually with vomiting and up to dehydration [14].

Severe watery diarrhea caused by ETEC results from release of a heat-labile toxin (LT), a heat-stable toxin (ST) or both. Pathogenicity is also dependent upon the expression of fimbriae or colonization factors (CFs) that act as adherence factors, allowing the organism to colonize the small intestine [15].

The LT is a protein multimer which shares many features and biological activity with cholera toxin, and both are considered as virulence factors and modulators of immune responses in mammalian species, including humans [4,14]. LT toxin in human is encoded by the eltA and eltB genes. [16].

Both animal assays and cell culture techniques were mainly used for detection of ETEC strains that required specific antibodies to demonstrate the presence of LT and/or ST enterotoxins but these methods were slow and labor-intensive. Enzyme-linked immunosorbent assays and co-agglutination immunoassays are considered attractive alternatives to the earlier methods as they are more rapid and easy to use. Further improvement in methods for the detection of ETEC gave rise to PCR assays which are more rapid, sensitive, and specific method used for ETEC diagnosis. The molecular detection and

typing by PCR has many advantages over conventional methods. It facilitates the detection and control of outbreaks caused by ETEC providing an important approach for the detection and analysis of many pathogens [17-20].

The aim of the present study is to determine the incidence of LT producing ETEC as a causative agent of acute diarrhea in children under 5 years in our hospital and to study the phenotypic characters of LT producing ETEC and finally to investigate the presence of the heat labile toxigenicity gene in isolated *E. coli* strains.

2. MATERIALS AND METHODS

2.1 Study Design and Population

This study was a prospective longitudinal study carried out on 100 children under 5 years, suffering from acute diarrhea without visible blood (persisting less than 14 days), admitted to Diarrhea and Malnutrition Unit of Pediatric Department, Tanta University Hospital from October 2013 to October 2015. Written informed consent was obtained from parents of all participants. Ethical approval for this study was provided by ethics and research committee of our institute. This study was conducted in the Medical Microbiology & Immunology Department, Faculty of Medicine, Tanta University. Children more than 5 years and those who received systemic antibiotic for at least 72 hours before admission were excluded from this study.

2.2 Sample Collection and Processing

All stool samples were collected in sterile containers, before antibiotic therapy was begun. The samples were transferred within one hour to the laboratory and were subjected to the following:

2.2.1 Phenotypic characterization of ETEC

2.2.1.1 Isolation and identification of E. coli

The specimens were cultured directly on MacConkey agar by streak plate method and incubated aerobically at 37ºC for 24 hours and then studied under direct light to observe the colony morphology (shape, size, surface texture, edge, elevation, colour and opacity). The organisms showing characteristic colony morphology of *E. coli* (2 to 3 mm pink lactose fermenting colonies) was repeatedly subcultured onto Eosin Methylene Blue (EMB) agar (*E. coli* appears as black colonies with a greenish-black metallic sheen) until the pure culture with homogenous colonies was obtained.

Further identification of the isolated organisms was done by microscopic examination using the Gram stain to determine the size, shape and arrangement of bacteria. *E. coli* are Gram negative bacilli, of variable size, arranged singly or in pairs, non sporing and non capsulated. Biochemical reactions were done using API 20 E (Biomerieux, France).

2.2.1.2 Detection of enterotoxigenic E. coli strains through using Phadebact R ETEC-LT test (Bactus AB Inc., Sweden)

The isolated *E. coli* from the studied samples were then subjected to Phadebact R ETEC-LT Test, which is a co-agglutination test. The ETEC-LT Reagent is composed of antibodies against highly purified LT enterotoxin, raised in rabbits and coupled to the protein A of non-viable *Staphylococci*. A drop of a cell extraction of suspended bacterial colonies from LT-producing *E. coli* strains was mixed with the ETEC-LT reagent. Specific antigens (if present) bind to corresponding antibodies on the surface of the *Staphylococci*. When a co-agglutination lattice is formed, it is visible to the naked eye and read within 1 min.

2.2.1.3 Antibiotic sensitivity testing of ETEC strains using Modified Kirby-Bauer disk diffusion method

E. coli strains positive for ETEC genes were analyzed for their antimicrobial susceptibilities by disk diffusion, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (5). The antibiotics analyzed were ampicillin (AMP; 10 µg disk), co-trimoxazole (SXT; 23.75/1.25 µg disk), tetracycline (TE; 30 µg disk), Ceftriaxone (CRO; 30 µg disk), chloramphenicol (C; 30 µg disk), ciprofloxacin (CIP; 5 µg disk), gentamicin (GTM; 10 µg disk) and imipenem (IPM; 10 µg disk).

Antimicrobial susceptibilities were tested by the disk diffusion method using Muller Hinton agar, and were interpreted according to National Committee for Clinical Laboratory Standards guidelines (CLSI 2014). *E. coli* ATCC 25922 was used as quality control strains.

2.2.2 Genotypic characterization of ETEC

This was done by detection of heat-labile toxogenecity gene *(elt)* in isolated *E. coli* strains by PCR.

2.2.2.1 DNA extraction

DNA extraction from harvested bacterial cells was performed using Thermo Scientific Gene JET Genomic DNA Purification Kit #K0721 (Thermo Fisher Scientific Inc., USA) with protocol provided by the manufacturer.

2.2.2.2 PCR amplification

LT primers targeting *elt* gene (Sigma inc., Egypt) LT.F-5´ GCACACGGAGCTCCTCAGTC-3´ and LT.R-5´ CCTTCATCCTTTCAATGGCTTT 3´ were used [21]. Reference ETEC (SSI Diagnostica, Denmark) and non-ETEC strains were used as positive and negative controls. The PCR reaction was performed in a 50 µl reaction volume containing 25 µl Maxima® Hot Start PCR Master Mix (2X) (Thermo Fisher Scientific Inc., USA). 0.5 µM of each primer (forward and reverse),1 µg template DNA and to 50 µl nuclease free water. The samples were gently vortexed and briefly centrifuged to collect all drops to the bottom of the tube. The samples were overlaid with mineral oil (Biomerieux) and placed in the thermal cycler (Thermo Fisher Scientific Inc., USA). After an initial denaturation at 95℃ for 4 min, the cycling conditions were 40 cycles of 95℃ for 30s, 59℃ for 30s, and 72℃ for 1 min, followed by a final elongation at 72℃ for 5 min. Fifteen microliters of PCR product of each sample as well as positive control, negative control and DNA marker was electrophoresed through a 2% agarose gel stained with ethidium bromide and visualised under an UV illumination. Specimens with positive bands at 305 bp were considered to be positive LT producing *E. coli.*

2.3 Statistical Analysis

Results were tabulated and statistical analysis was performed with Statistical Package for Social Science (SPSS version 13). Comparison between the studied groups was performed with Chi-square testing (X^2). P values of <0.05 were considered statistically significant.

3. RESULTS

One hundred children under 5 years of age, 38 females and 62 males suffering from acute diarrhea were included in this study. The results of stool culture of the studied cases are shown in Table 1. *E. coli* was isolated from 94 cases constituting 94% and out of the (94) isolated *E. coli* strains, there were 8 (8.5%) LT producing ETEC. The incidence of LT producing ETEC among all studied children with diarrhea was 8%. While 6 children did not reveal the presence of *E. coli* constituting 6%.

Table 1. Incidence of LT producing ETEC among isolated organisms from children under 5 years suffering from acute diarrhea

Types of isolated organisms		Cases (n=100)	
		N	%
E.coli (n=94)	LT producing ETEC	8	8.5
	Non LT producing *E. coli*	86	91.5
organisms other than *E.coli* (n=6)		6	6

Regarding the results of laboratory tests used for the detection of LT producing ETEC it was found that PCR is significantly a more sensitive method when compared with Phadebact test (P = 0.0001). The Phadebact test detected 6 LT producing ETEC cases out of 8 cases (showed 2 false negative cases) constituting 75% of all ETEC cases detected among studied cases showing 75% sensitivity when compared to PCR. While PCR detected 8 cases of LT producing ETEC constituting 100% of all ETEC cases detected among studied cases showing 100% sensitivity when compared to Phadebact R test. The Phadebact test revealed the presence of 6 LT producing ETEC strains (The test was strong positive in 4 of them and weak positive in 2 strains). Those 2 weak positive strains gave positive results for the presence of elt toxigenicity gene when they were examined by PCR.

There is a significant difference between sensitivity of both methods of detection of LT producing ETEC. (P value <0.05) (Table 2).

Distribution of isolated organisms from the studied children with acute diarrhea in relation to their sex is illustrated in Table 3. Five out of eight LT producing ETEC strains were isolated from male children constituting 13.2% of total of 38 studied males (62.5% of total isolated LT producing ETEC as shown in Fig. 1). While the other 3 strains were isolated from female children constituting 4.8% of total of 62 studied females (37.5% of total isolated LT producing ETEC as shown in Fig. 1).

Table 2. Results of laboratory tests use for detection of LT producing ETEC from the isolated microorganisms from the studied cases

Laboratory test		Positivity of detecting LT producing ETEC from the study samples (n=8)	
		N	%
Phadebact test	Strong +ve	4	50.0
	Weak +ve	2	25.0
	False –ve	2	25.0
PCR	+ve	8	100
	-ve	0	0
X2		17.130	
P		0.0001*	

Table 3. Distribution of isolated organisms from the studied children with acute diarrhea in relation to their sex

Isolated organisms	Sex of the studied children (n=100)						P
	Males		Females		Total		
	N	%	N	%	N	%	
• LT producing ETEC	5	13.2	3	4.8	8	8.0	0.253
•Other isolated organisms	33	86.8	59	95.2	92	92.0	
Total	38	38.0	62	62.0	100	100	

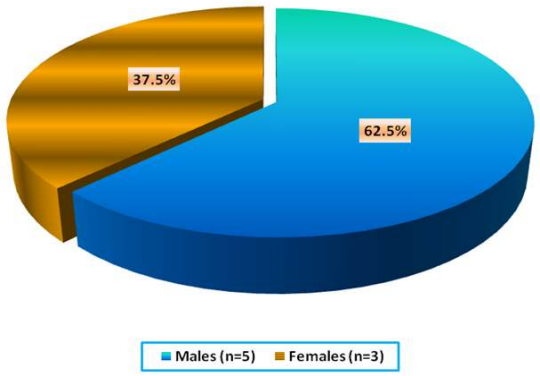

Fig. 1. Sex of the studied children suffering from acute diarrhea with isolated LT producing ETEC (n=8)

There was no significant sex difference detected between isolated LT producing ETEC cases and cases with other organisms (non LT producing *E. coli* and organisms other than *E. coli*) ($p > 0.05$).

Table 4 shows that 7 LT producing ETEC strains were isolated from children ≤ 2 years constituting 10.3% of total of 68 studied children below 2 years (87.5% of total isolated LT producing ETEC as shown in Fig. 2). And only one strain was isolated from children > 2 years constituting 3.1% of total of 32 studied children above 2 years (12.5% of total isolated ETEC as shown in Fig. 2).

Distribution of isolated organisms from the studied children with acute diarrhea in relation to their clinical symptoms and nutritional status is illustrated in Table 5. There was symptom of fever and vomiting in diarrheal cases caused by non- LT producing ETEC organisms when compared with LT producing ETEC diarrheal cases ($p < 0.05$). On the other hand, there was no sign of malnourishment or underweight in diarrheal cases caused by LT producing ETEC organisms when compared with non-LT producing ETEC diarrheal cases ($p > 0.05$).

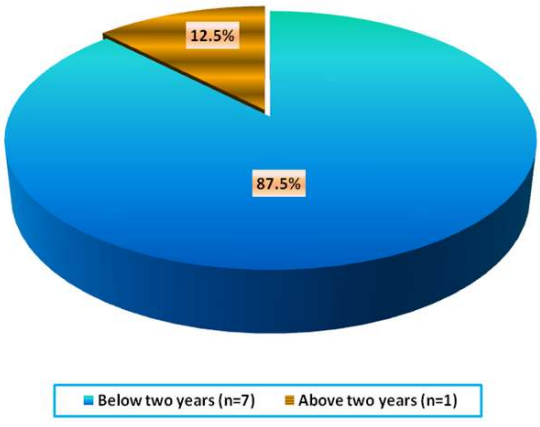

Fig. 2. Age of the studied children suffering from acute diarrhea with isolated LT producing ETEC (n=8)

Table 6 shows the distribution of LT producing ETEC and other isolated organisms from the studied cases with acute diarrhea in relation to the type of feeding. Fifty percent of total isolated ETEC strains were isolated from completely weaned children constituting 8.5% of total 47 studied completely weaned children. LT producing ETEC strains were isolated from completely weaned children (50% of total isolated ETEC) when compared to combined fed children (37.5% of total isolated ETEC) and exclusively breast fed (12.5% of total isolated ETEC) (p=0.002).

The seasonality of LT producing ETEC was mainly during summer months and early autumn with a peak during mid-summer months (Fig. 3).

The antibiotics susceptibility testing results of the isolated LT producing ETEC showed that resistance of LT producing ETEC strains was found against SXT (75%), ampicillin (62.5%), gentamycin (62.5%) and tetracycline (50%). While sensitivity to both ciprofloxacin and imepinem was (100%), ceftriaxone (87.5%) and chloramphenicol (62.5%) (Table 7).

4. DISCUSSION

ETEC has shown to be an important cause of diarrheal disease in the developing world especially in children [13]. However, testing for its presence is generally beyond the scope of most hospital microbiological laboratories.

In this study, we reported the incidence of LT producing ETEC in isolates recovered from children seeking care at Tanta University Hospitals. An overall incidence of LT producing ETEC was found to be (8%) of all diarrhea cases during study period. The incidence of LT

Table 4. Distribution of isolated organisms (LT producing ETEC and other isolated organisms) from the studied children with acute diarrhea in relation to their age (n=100)

Isolated organisms	Age of the studied children (n=100)						P
	≤ 2 year		< 2 years		Total		
	N	%	N	%	N	%	
• LT producing ETEC	7	10.3	1	3.1	8	8.0	0.430
• Other isolated organisms	61	89.7	31	96.9	92	92.0	
Total	68	68.0	32	32.0	100		

Table 5. Distribution of isolated organisms from the studied children with acute diarrhea in relation to their clinical symptoms and nutritional status

Isolated organisms	Isolated organisms from the studied children (n=100)						P
	LT producing ETEC diarrheal cases (n=8)		Non- LT producing ETEC diarrhea (n=92)		Total (n=100)		
	N	%	N	%	N	%	
• Vomiting	3	37.5	86	93.5	89	89.0	0.0003*
• Fever	4	50.0	92	100	96	97.0	0.0001*
• Malnourishment & under-weight	1	12.5	18	19.4	6	6.0	0.54

Table 6. Distribution of LT producing ETEC and other isolated organisms from the studied cases with acute diarrhea in relation to the type of feeding

Isolated organisms	Type of feeding of the studied children (n=100)						P
	Exclusive breast feeding		Combined feeding		Completely weaned		
	N	%	N	%	N	%	
• LT producing ETEC	1	3.7	3	11.5	4	8.5	**0.002***
•Other isolated organisms	26	96.3	23	88.5	43	91.5	
Total	27	27.0	26	26.00	47	47.0	

Table 7. The antibiogram of the isolated LT producing ETEC strains

Antibiotic disc	Disc content	The studied cases (no=8)					
		S		I		R	
		no.	%	no.	%	no.	%
Ampicillin (AMP)	10 µg	3	37.5	-	-	5	62.5
Ceftriaxone (CRO)	30 µg	7	87.5	-	-	1	12.5
Ciprofloxacin (CIP)	5 µg	8	100	-	-	-	-
Gentamicin (CN)	10 µg	1	12.5	2	25	5	62.5
Co-trimoxazole (SXT)	23.75/1.25 µg	1	12.5	1	12.5	6	75
Chloramphenicol (C)	30 µg	5	62.5	-	-	3	37.5
Tetracycline (TE)	30 µg	1	12.5	3	37.5	4	50
Imipenem (IPM)	10 µg	8	100	-	-	-	-

producing ETEC strains in this work was almost similar to that reported in another Egyptian study by Mahdy et al. [22] who investigated 70 children ranged from 3 months up to 5 years at outpatient clinic, Abu El-Reesh pediatric hospital, reported 5 ETEC strains out of 63 *E. coli* isolates (8%).

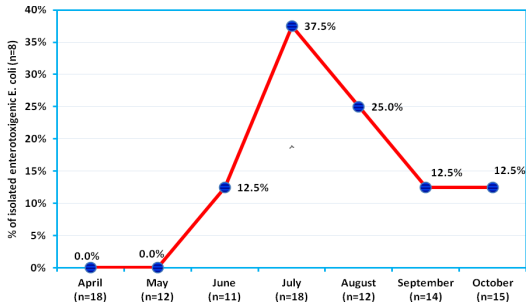

Fig. 3. Seasonality of the isolated LT producing ETEC from the studied children with acute diarrhea (n=8)

Our results are also consistent with earlier studies in developing countries as a previous report by Rivera et al. [23] who identified a prevalence of ETEC of 5.3% in children with diarrhea between 2 and 24 months of age. Also with another study by Gonzales et al. [24] where 9% of the diarrhea cases were due to ETEC during a 4 years period study. Moreover, another study in Brazil by Nunes et al. [25] reported that the prevalence of ETEC was (9.2%).

On the other hand, Shaheen et al. [26] who investigated children of Abu Homos District, Beheira Governorate, Egypt found that 27% of diarrheal episodes were associated with the excretion of ETEC. Abu Elyazeed et al. [27] found 125 episodes of ETEC diarrhea from 242 children less than 3 years (51%). Also higher ETEC prevalence was shown in earlier studies in Argentina and Nicaragua (18.3% and 38%, respectively) [28,29] suggesting that there are clear differences in the frequencies of ETEC infection in different countries. These differences may be related to the age of the population and the type of study (prospective versus retrospective and community-based versus hospital-based study). The comparatively low incidence of ETEC in this study and other Egyptian studies may be due to the fact that only children who are ill enough are brought to the hospital for treatment so we may lack information on milder illnesses not requiring medical attention and thus underestimate the total ETEC diarrhea incidence in this population.

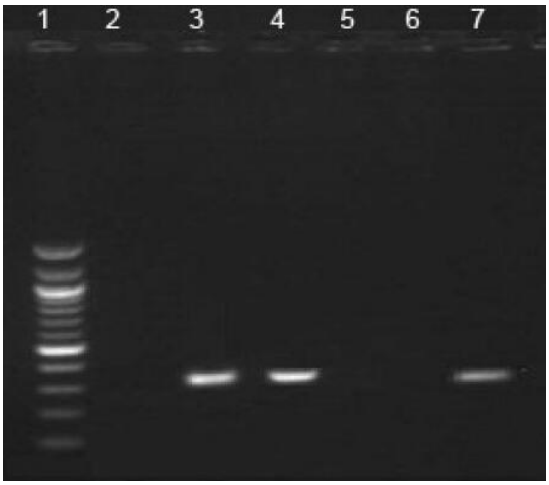

Fig. 4. Gel electrophoresis pattern of amplified LT producing ETEC PCR products

Lane 1: molecular marker.
Lane 2: negative control.
Lane 3: positive control.
Lane 5, 6: negative samples for 305 bp.
Lane 4, 7: positive samples for 305 bp.

The low proportion of detected ETEC infections (8%) may be due to the fact that the aim of the study was to isolate LT positive strains only. Previous studies in Egypt have shown that ST

positive ETEC strains are more commonly associated with outbreaks and diarrheal episodes [13,27] However, these studies detected enterotoxins by phenotypic and not molecular methods, and therefore, the real frequency of LT may have been underestimated.

Although, Qadri et al. [13] stated that ETEC expressing LT only have been considered less important as pathogens, since they are frequently isolated (than the other two toxin types) from healthy persons than from patients. However, they decided that LT-producing ETEC cannot be excluded as a highly pathogenic agent, given that they may have been isolated from sick patients with severe dehydrating diarrhea. Moreover, in another study Qadri *et al*. [30] explained that this discrepancy might be due to seasonality for toxin types and a high predominance of that ST ETEC strains were usually seen in the spring.

During the study, rate of isolation of mixed bacterial infections was considered very low (3%). Meanwhile, Shaheen et al. [15] reported that ETEC was the principal identified cause of diarrhea among their study children, with an incidence eight times more than that of *rotavirus*, *astrovirus*, or *Shigella*. They also stated that coinfection with ETEC and other enteric pathogens is common. Also, Qadri et al. [13] stated that the incidence of mixed infections seems to increase with age and fewer co-pathogens were seen in infants than in older children and adults with ETEC diarrhea.

The incidence of ETEC diarrhea in our sample was not significantly affected by age or sex of studied children. This was in agreement with the results by Das et al. [31] who found that ETEC was commonly isolated from the studied infants less than 2 years of age, accounting for about 70% of the first diarrheal episodes. Furthermore, during a study in Bangladesh by Qadri et al. [13] including children less than 9 years of age, the proportion of children infected with ETEC was higher in children less than 2 years of age, with the prevalence decreasing as the age of the children increased.

In this study, almost 30% of the children with ETEC infection suffered from vomiting while fever was reported in about 50% of children shedding ETEC strains. However, presence of fever and vomiting in ETEC cases was of no significance when compared with other non-ETEC diarrheal cases and didn't lead to significant malnourishment in ETEC cases.

Similarly, Shaheen et al. [15] did not demonstrate any correlation between clinical symptoms and ETEC isolated from children seeking hospital care. They explained that additional factors may dispose these children to malnutrition including other enteric infections as well as their low socioeconomic living conditions and contaminated environment.

Nevertheless, a previous report of ETEC isolates recovered from hospitalized infants by Evans et al. [32] found the ETEC infection to be commonly associated with fever and vomiting. The observation of severe in some studies may be attributed to the presence of strains that are more virulent because of the presence of additional virulence attributes.

Qadri et al. [30] stated that ETEC diarrhea appeared to be associated with the poor nutritional status of the children. Thus, those children who had experienced one or more episodes of diarrhea due to ETEC as a single pathogen were significantly more malnourished than those without any episode of ETEC disease.

Concerning the effect of breast-feeding on diarrhea, we observed that the rate of ETEC diarrhea was significantly lower in exclusively breast fed children (3.7%) when compared to combined fed (11.5%) or completely weaned ones (8.5%), which suggests an overall protective effect of breast-feeding.

Similarly, studies in Bangladesh [30] and Mexico [33] have suggested that breast-feeding is associated with a reduced risk of ETEC diarrhea in infancy. However, they have also shown that this protection does not last over the first 2 to 3 years of life.

In our study, ETEC majority of infections were occurring from June to October, similar to seasonal patterns found for LT strains in previous study that was carried out in Egypt [34] and similar to Shaheen et al. [26] time plots which showed that diarrheal episodes caused by clonally related ETEC strains emerged in temporal clusters mostly during the warm season in Egypt.

Similarly, Estrada-Garcia et al. [35] reported that ETEC-associated diarrhea episodes were identified from July to October, with a clear peak

in August, during the summer rainy season in Mexico City.

On the other hand, Qadri et al. [30] reported peaks of ETEC infections in the early spring and late autumn months. Isolation of ETEC from diarrheal children followed a very characteristic biannual seasonality with two separate peaks, one at the beginning of the hot season, that is, the spring, and another peak in the autumn months, but it remains endemic all year.

Whereas, Gonzales et al. [24] observed a small seasonal peak of ETEC cases in May and coincided with the peak of rotavirus infections. This is an unusual seasonality for ETEC strains, which usually have a peak in the number of infections during the transition to the warm-rainy seasons in the tropics. Such seasonality was explained by Qadri et al. [13] due to climate and spread by environmental factors. As the atmospheric temperature increases, there is increased growth of bacteria in the environment and this continues in the summer months. Furthermore, with the advent of rains, the surface water can thus become heavily contaminated.

Many methods for detection of LT-producing ETEC have been reported, but most of them have some disadvantages as well as advantages. During this study, Phadebact test proved to be simple, easy and less time consuming method for detection of ETEC.

Moreover, Ronnberg et al. [36] found that results of co-agglutination test are so promising that the test should be evaluated in developing countries and in hospital laboratories in industrialized countries as a new test procedure in studies of travelers' diarrhea and in diarrhea of unknown origin, especially in young children.

Also, Wadstrom et al. [37] believed that in future studies using this test in diagnostic laboratories on primary stool cultures would be an advantage to avoid the burdensome extra step of growing each isolate from stool cultures in liquid culture before performing the immunoassays as described in radioimmunoassay and ELISA test procedures.

Similarly, Sen et al. [38] reported that the modified *Staphylococcal* Co-A test was the best in terms of simplicity. A large number of *E. coli* isolates could be screened for LT production within a short time, using ordinary inexpensive laboratory equipment and reagents.

On the other hand, Czirbk et al. [39] compared the Oxoid ELISA test with the Phadebact LT test, the former proved to be more sensitive but needs more complex procedure, special formulated media, shaking culture, experienced laboratory personnel and it is more time consuming than Phadebact test.

In the present study, we provide evidence that routine employment of PCR-based reagents provide improved efficiency of detecting ETEC. The corresponding gene was always detected in phenotypically positive strains with the genotypic method (100%), and consequently, the genotypic assay seemed to have a high level of sensitivity for detecting the gene in strains with the corresponding phenotypic expression. On the other hand, very few strains that were positive by the genotypic methods were phenotypically negative (25%) or weakly positive (25%).

This can be explained by the possibility that either the *elt* gene is present as a silent gene or, alternatively, that the phenotypic expression level of LT in certain strains may be very low to be considered, since its expression might have been lost during subculture or storage but present in the initial clinical isolate.

Also, Rivera et al. [23] reported that PCR was superior to enzyme immunoassay techniques. Their study results showed that 37% of PCR-positive strains were negative by GM1-ELISA explaining that the used for detection of the enterotoxins are dependent on the invitro production of these factors, whereas molecular techniques can detect the presence of the genes.

Similarly, Sjoling et al. [40] have shown PCR to be an efficient method for the detection of LT and ST, having better sensitivity and specificity than other methods such as ELISA.

The results of this study suggest that antimicrobial resistance is widespread among potentially ETEC strains. Most of tested strains were sensitive to ceftriaxone (87.5%) while high resistance to SXT (75%), ampicillin (62.5%), gentamycin (62.5%) and tetracycline (50%) was demonstrated. These results are, to a great extent, similar to other study in Egypt by Badri et al. [41] who showed that the occurrence of antibiotic resistance among *E. coli* isolates from patients with acute diarrhea was 68.2% and 57.2% for ampicillin and SXT, respectively. Similarly El-Rami et al. [42] demonstrated that

ETEC strains from Egypt are routinely resistant to ampicillin and SXT.

Also, Wang et al. [43] reported high resistance to antibiotics, especially ampicillin, SXT, streptomycin, tetracycline and emerging resistance to cephalosporin in 37% of diarrheagenic *E. coli*.

Barati et al. [44] showed high multidrug resistance among toxigenic *E. coli* isolated from enteritis cases to SXT and ampicillin. Also, Rivera et al. [23] found a high level of antibiotics resistance in the Bolivian ETEC strains explaining that this multi-resistance of ETEC might emerge to classical antibiotics such as ampicillin, tetracycline, and SXT since they have been widely used in Bolivia during the past few years.

In our study, all ETEC strains were 100% sensitive to quinolones. This can be explained by limited use of quinolones in children. Similarly Guerra et al. [21] reported 100% sensitivity to ciprofloxacin. While, Rodrigues et al. [45] observed that 20% of the ETEC strains isolated from patients with diarrhea during their study were highly resistant to quinolones and majority of the ETEC investigated in that study was resistant to several other antibiotics as well. That multiple antibiotic resistances may be acquired through mobile genetic elements such as plasmids and transposons which play an essential role in facilitating the transfer of the resistance genes. Other factors can also contribute in increasing the numbers of resistant bacteria in the community such as regional consumption of antibiotics and open access to antibiotics from local pharmacies leading to significant antibiotic misuse.

5. CONCLUSION

This study demonstrates that the incidence of diarrheal disease in children less than five years caused by LT producing ETEC was (8%). The incidence was not affected by sex or age. It was significantly lower in breast fed infants with a seasonal peak during summer and early autumn. Rapid screening test (co-agglutination) is a useful addition to laboratory diagnostic procedures in regions where ETEC is endemic. PCR has high level of sensitivity and able of detecting both expressed and silent genes. Sensitivity of isolates to ceftriaxone in this study suggests that this antibiotic is a rational choice for the treatment of prolonged childhood diarrhea in which ETEC is of a primary etiologic consideration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kosek M, Bern C, Guerrant RL. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull. W.H.O. 2003;81:197-204.
2. Bryce J, Boschi-Pinto C, Shibuya K, Black, RE. WHO child health epidemiology reference group. WHO estimates of the causes of death in children. Lancet. 2005;365:1147-1152.
3. O'Ryan M, Prado V, Pickering LK. A Millennium update on pediatric diarrheal illness in the developing world. Semin Pediatr Infect Dis. 2005;16(2):125-136.
   DOI: 10.1053/j.spid.2005.12.008
4. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998;11:142-201.
5. Guion CE, Ochoa TJ, Walker CM, Barletta F, Cleary TG. Detection of diarrheagenic *Escherichia coli* by use of melting-curve analysis and real-time multiplex PCR. J Clin Microbiol. 2008;46:1752-1757.
   DOI: 10.1128/JCM.02341-07
6. World Health Organization. New frontiers in the development of vaccines against enterotoxigenic (ETEC) and enterohaemorrhagic (EHEC) *E. coli* infections. Weekly Epidemiol Rec. 1999; 13:98–100.
7. Huilan S, Zhen LG, Mathan MM, Mathew MM, Olarte J, Espejo R, Maung UK, Ghafoor MA, Khan MA, Sami Z. Etiology of acute diarrhoea among children in developing countries: A multicentre study in five countries. Bull. W.H.O. 1991;69: 549–555.
8. Sack RB, Gorbach SL, Banwell JG, Jacobs B, Chatterjee BD, Mitra RC. Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like disease. J Infect Dis. 1971;123:378–85.
9. Nataro JP, Deng Y, Cookson S, Cravioto A, Savarino SJ, Guers LD, Levine MM, Tacket CO. Heterogeneity of entero-aggregative *Escherichia coli* virulence

demonstrated in volunteers. J Infect Dis. 1995;171(2):465–8.
DOI: 10.1093/infdis/171.2.465

10. Ericsson CD. Travellers' diarrhoea, in principles and practice of travel medicine, Second Edition (ed Zuckerman JN), Wiley-Blackwell, Oxford, UK; 2013.
DOI: 10.1002/9781118392058.ch13
11. Lanata CF, Mendoza W, Black RE. Improving diarrhea estimates. WHO, Geneva, Switzerland; 2002.
Available:http://www.who.int/child_adolescent_health/documents/pdfs/improving_diarrhoea_estimates.pdf
(Accessed on 22 February 2016)
12. Kothary MH, Babu US. Infective dose of food borne pathogens in volunteers. A review. J. Food Sci. 2001;21(1):49–73.
DOI: 10.1111/j.1745-4565.2001.tb00307.x
13. Qadri F, Svennerholm A-M, Faruque AS, Sack RB. Enterotoxigenic *Escherichia coli* in developing countries: Epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev. 2005;18:465–483.
DOI: 10.1128/CMR.18.3.465-483.2005
14. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2:123–140.
DOI: 10.1038/nrmicro818
15. Shaheen HI, Abdel Messih IA, Klena JD, Mansour A, El-Wakkeel Z, Wierzba TF, Sanders JW, Khalil SB, Rockabrand DM, Monteville MR, Rozmajzl PJ, Svennerholm AM, Frenck RW. Phenotypic and genotypic analysis of enterotoxigenic *Escherichia coli* in samples obtained from Egyptian children presenting to referral hospitals. J Clin Microb. 2009;47(1):189–197.
DOI: 10.1128/JCM.01282-08
16. Lasaro MA, Rodrigues JF, Mathias-Santos C, Guth BE, Balan A, Sbrogio-Almeida ME, Ferreira LCS. Genetic diversity of heat-labile toxin expressed by enterotoxigenic *Escherichia coli* strains isolated from humans. J. Bacteriol. 2008;190(7):2400–2410.
DOI: 10.1128/JB.00988-07
17. Schultsz, C, Pool GJ, van Ketel R, de Wever B, Speelman P, Dankert J. Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. J Clin Microbiol. 1994;32:2393-2397.
18. Yavzori M, Porath N, Ochana O, Dagan R, Orni-Wasserlauf R, Cohen D. Detection of enterotoxigenic *Escherichia coli* in stool specimens by polymerase chain reaction. Diagn Microbiol Infect Dis. 1998;31:503-509.
19. Reischl U, Youssef MT, Kilwinski J, Lehn N, Zhang WL, Karch H, Strockbine NA. Real-time fluorescence PCR assays for the detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. J Clin Microbiol. 2002;40:2555-2565.
DOI: 10.1128/JCM.40.7.2555-2565.2002
20. Reischl U, Youssef MT, Wolf H, Hyytia-Trees E, Strockbine NA. Real-time fluorescence PCR assays for detection and characterization of heat-labile I and heat-stable I enterotoxin genes from enterotoxigenic *Escherichia coli*. J Clin Microbiol. 2004;42(9):4092-4100.
DOI: 10.1128/JCM.42.9.4092-4100.2004
21. Guerra JA, Herazo YC, Arzuza O, Gómez OG. Phenotypic and genotypic characterization of enterotoxigenic E.coli clinical isolates from northern Colombia, South America. Biomed Res Int. 2014;236260:1-11.
DOI: 10.1155/2014/236260
22. Mahdy HM, Fareid MA, Negm ME. Toxin of enterotoxigenic *E. coli* causing gastro-enteritis in children. J of Appl Scienc Res. 2010;6(6):756-776.
23. Rivera FP, Ochoa TJ, Maves RC, Bernal M, Medina AM, Meza R, Barletta F, Mercado E, Ecker L, Gil AI, Hall ER, Huicho L, Lanata CF. Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from Peruvian children. J Clin Microbiol. 2010;48(9):3198–3203.
DOI: 10.1128/JCM.00644-10
24. Gonzales L, Sanchez S, Zambrana S, Iniguez V, Wiklund G, Svennerholm AM, Sjöling A. Molecular characterization of enterotoxigenic *Escherichia coli* isolates recovered from children with diarrhea during a 4-year period (2007 to 2010) in Bolivia. J Clin Mic. 2013;51(4):1219–1225.
DOI: 10.1128/JCM.02971-12
25. Nunes MD, Penna FJ, Franco RT, Mendes EN, Magalhaes PP. Enterotoxigenic *Escherichia coli* in children with acute diarrhea and controls in Teresina/PI, Brazil: distribution of enterotoxin and colonization factor genes. J Appl Microbiol. 2011;111(1):224–232.
DOI: 10.1111/j.1365-2672.2011.05031.x

26. Shaheen H, Khalil S, Rao M, Abu Elyazeed R, Wierzba TF, Peruski LF, Putnam S, Navarro A, Morsy BZ, Cravioto A, Clemens JD, Svennerholm AM, Savarino SJ. Phenotypic profiles of enterotoxigenic *Escherichia coli* associated with early childhood diarrhea in rural Egypt. J. Clin. Microbiol. 2004;42(12): 5588–5595.
DOI: 10.1128/JCM.42.12.5588-5595.2004
27. Abu-Elyazeed R, Wierzba T, Mourad A, Peruski LF, Kay BA, Rao M, Churilla AM, Bourgeois AL, Mortagy AK, Kamal SM, Savarino SJ, Campbell JR, Murphy JR, Naficy A, Clemens JD. Epidemiology of enterotoxigenic *Escherichia coli* diarrhea in a pediatric cohort in a periurban area of lower Egypt. J Infect Dis. 1999;179(2): 382-389.
DOI: 10.1086/314593
28. Viboud GI, Jouve MJ, Binsztein N, Vergara M, Rivas M, Quiroga M, Svennerholm A. Prospective cohort study of enterotoxigenic *Escherichia coli* infections in Argentinean children. J Clin Microbiol. 1999;37(9): 2829–2833.
29. Paniagua M, Espinoza F, Ringman M, Reizenstein E, Svennerholm AM, Hallander H. Analysis of incidence of infection with enterotoxigenic *Escherichia coli* in a prospective cohort study of infant diarrhea in Nicaragua. J Clin Microbiol. 1997;35(6):1404–1410.
30. Qadri F, Saha A, Ahmed T, Al Tarique A, Begum YA, Svennerholm A. Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh. Infect Immun. 2007;7(8):3961–3968.
DOI: 10.1128/IAI.00459-07
31. Das JK, Tripathi A, Ali A, Hassan A, Dojosoeandy C, Bhutta ZA. Vaccines for the prevention of diarrhea due to cholera, shigella, ETEC and rotavirus. BMC Public Health. 2013;13(3):S11.
DOI: 10.1186/1471-2458-13-S3-S11
32. Evans DJ, Evans DG. Three characteristics associated with enterotoxigenic *Escherichia coli* isolated from man. Infect Immun. 1973;8(3):322-328.
33. Long KZ, Wood JW, Vasquez GE, Weiss KM, Mathewson JJ, de la Cabada FJ, DuPont HL, Wilson RA. Proportional hazards analysis of diarrhea due to enterotoxigenic *Escherichia coli* and breast feeding in a cohort of urban Mexican children. Am. J. Epidemiol. 1994;139(2): 193–205.
34. Rao MR, Abu-Elyazeed R, Savarino SJ, Naficy AB, Wierzba TF, Abdel-Messih I, Shaheen H, Frenck RW Jr, Svennerholm AM,Clemens JD. High disease burden of diarrhea due to enterotoxigenic *Escherichia coli* among rural Egyptian infants and young children. J Clin Microbiol. 2003;41(10):4862–4864.
DOI: 10.1128/JCM.41.10.4862-4864.2003
35. Estrada-Garcia T, Lopez-Saucedo C, Thompson-Bonilla R, Abonce M, Lopez-Hernandez D, Santos JI, Rosado JL, DuPont HL, Long KZ. Association of diarrheagenic *Escherichia coli* pathotypes with infection and diarrhea among Mexican children and association of atypical enteropathogenic *E. coli* with acute diarrhea. J Med Micro. 2009;47(1):93–98.
DOI: 10.1128/JCM.01166-08
36. Ronnberg B, Wadstrom T. Rapid detection by a coagglutination test of heat-labile enterotoxin in cell lysates from blood agar-grown *Escherichia coli*. J Clin Microb. 1983;17(6):1021-1025.
37. Wadstrom T, Soderlind O, Mollby R, Wretlind B. Release of heat-labile enterotoxin from cells of *Escherichia coli* strain 853/67. IRCS Libr. Compend. 1974;2:1312.
38. Sen D, Saha M, Pal S. Evaluation of three simple and rapid immunological tests for detection of heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. J Clini Micr. 1984;19(2):194-196.
39. Czirbk E, Semjen G, Steinruck H, Herpay M, Milch H, Nyomárkay I, Stverteczky Z, Szeness A. Comparison of rapid methods for detection of heat-labile (LT) and heat-stable (ST) enterotoxin in *Escherichia coli*. J Med Microbiol. 1992;36(6):398-402.
DOI: 10.1099/00222615-36-6-398
40. Sjoling A, Wiklund G, Savarino S, Cohen D, Svennerholm AM. Comparative analyses of phenotypic and genotypic methods for detection of enterotoxigenic *Escherichia coli* (ETEC) toxins and colonization factors. J Clin Microbiol. 2007;45(10):3295–3301.
DOI: 10.1128/JCM.00471-07
41. Badri S, Fassouane A, Bouslikhane M, Filliol I, Hassar M, Cohen N. Relationship between susceptibility to antimicrobials and virulence factors in *Escherichia coli*

isolated from food in Morocco. Int J Food Saf. 2009;11(2):98-101.

42. El-Rami FE, Rahal EA, Sleiman FT, Abdelnoor AM. Identification of virulence genes among antibacterial-resistant *Escherichia coli* isolated from poultry. Association between resistance and virulence genes. Adv Stu Biol. 2012;4(8): 385–396.
43. Wang Q, Wang S, Beutin L, Cao B, Feng L, Wang L. Development of a DNA microarray for detection and serotyping of enterotoxigenic *Escherichia coli*. J Clin Microb. 2010;48(6):2066–2074. DOI: 10.1128/JCM.02014-09
44. Barati S, Boniadian M, Habibian R, Jostejou T. Antibiotic resistance of enterotoxigenic and enteroaggregative *Escherichia coli* isolated from gastroenteritis cases. Asian J Biomed and Pharmac Sci. 2012;2(14):54-58.
45. Rodrigues JF, Mathias-Santos C, Sbrogio-Almeida ME, Amorim JH, Cabrera-Crespo J, Balan A, Ferreira LC. Functional diversity of heat-labile toxins (LT) produced by enterotoxigenic *Escherichia coli*: Differential enzymatic and immunological activities of LT1 (hLT) AND LT4 (pLT). J Biol Chem. 2011;286(7):5222–5233. DOI: 10.1074/jbc.M110.173682.

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