Multiplex PCR as emerging technique for diagnosis of enterotoxigenic *E. coli* isolates from pediatric watery diarrhea.

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Abstract Diarrhea continues to be one of the most common causes of morbidity and mortality among travelers and residents of developing countries especially infants and children. The aim of this study was to isolate *E. coli* from cases of diarrhea in children. A case-control study was conducted in 150 samples from children younger than fifteen years of age in Egypt (135 cases and 15 controls). Diagnosis of infection with this subclass of *E. coli* can be performed with conventional biochemical reactions and API 20E system. 87 bacterial isolates (*E coli*), 22 isolates (*Klebsiella* spp), 4 isolates (*Serratia marcescens*), *Enterobacter* spp (3 isolates) and 9 isolates (non lactose fermentation species) were obtained. There is significant effect between age groups and the percentage of isolated species ($P > 0.05$) but there is no significance for sex, fever and duration of diarrhea and isolates from each species ($P < 0.05$). The presence of genes encoding a heat-labile toxin (LT) and a heat-stable toxin (ST) was detected in fifty *E. coli* isolates by multiplex PCR. The result showed that, seventeen (34%) enterotoxigenic *E. coli* (ETEC) strains were found. Ten strains (20%) expressed ST, two (4%) expressed LT and five (10%) expressed LT&ST. This study supports the fact that ETEC is still a major cause of childhood diarrhea in Egypt, and that measures to prevent such infections are needed in developing countries.

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1. Introduction

Diarrheal disease (DD) is a serious public health problem in developing countries, and it remains a major cause of morbidity and mortality in children (Guerrant *et al*., 2002 and Mulcahy, 2013). In this age group it is estimated that 1.5 to 2.5 million deaths occur annually due to DD, that is, about 10 deaths every minute (Thielman and Guerrant, 2004). Among all etiological agents implicated in the causation of childhood diarrhoea, intestinal *Escherichia coli* pathogens are believed to be the second most frequent pathogens after rotavirus (Clarke 2001). The identification of *E. coli* pathotypes in association with diarrhoea has been limited in many developing countries because conventional microbiological testing is unable to distinguish between normal flora and pathogenic strains of *E. coli* (Persson *et al*., 2011; Vilchez *et al.*, 2014).

Six main groups of *E. coli* strains have been defined as enteric pathogens on the basis of their pathogenic mechanisms: the classical enteropathogenic strain (EPEC); enterotoxigenic (ETEC) that produce heat labile & heat stable enterotoxins; enteroinvasive strain (EIEC) that mimic *Shigella* strains in their ability to invade & multiply within intestinal epithelial cells; enterohemorrhagic (EHEC) strain that cause hemorrhagic colitis or produce shiga-like toxins; enteroaggregative (EAggEC) and diffusely adhering (DAEC) *E. coli* (Nataro and Kaper, 1998; Kaper *et al*., 2004; Roy *et al*., 2014).

Enterotoxigenic *E.coli* is primarily water born pathogen associated with diarrhea of children & travelers by colonizing the small intestine via fimbrial adhesive factors & express secretogenic enterotoxins of heat labile (LT) which is structurally similar to cholera toxin and /or heat stable (ST) which is now known to be a group of structurally similar small peptides that are also similar in chemical structure to the heat stable enterotoxins produced by several other bacteria (Nataro and Kaper, 1998; Kaper *et al*., 2004 and Kazi *et al.*, 2014).

Detection of enterotoxigenic *E. coli* strains have depended on demonstration of toxin production by specific bioassays and enzyme-liked immunosorbent assays or on the presence of the DNA sequences

encoding the toxins (Stacy-Phipps *et al*., 1995; Persson *et al*., 2011). Detection of enteric bacteria in fecal specimens from infected human and animals by PCR has been relatively insensitive because of the large amounts of inhibitors to PCR (Hornes *et al*., 1991; Newell *et al*., 2009). A multiplex polymerase chain reaction (PCR) assay has been reported to have greater sensitivity than other methods used to extract nucleic acid (Stacy-Phipps *et al*., 1995 ; Roshdy *et al*., 2006 and Shamsuzzaman *et al*. 2014). The aim of this study was to isolate *E.coli* from cases of diarrhea in children, and detect the presence of genes encoding a heat-labile toxin (LT) and a heat-stable toxin (ST) in order to detect all types of ETEC by multiplex PCR.

2. Material and Methods

Patients and Sampling

The samples included in this study were 150 samples, 15 as healthy control and 135 as patients suffered from diarrhea ranging from watery, loose, to bloody diarrhea. Most of patients were inpatients children at Shebin El koum Teaching Hospital. Others were from Hussein Hospital, Sayed Galal Hospital, Nasser Institute and Private Laboratories. This study was conducted over the period from October 2012 to December 2013.

Isolation and identification

Diarrheal stool samples were taken in the plastic cups and cultured directly on Mac Conky agar plates (about 1 h after collection), the inoculum on the plates was streaked out for discrete colonies with a sterile wire, then incubated at 37°C for 24 hours. Growing bacteria were isolated and identified by studying morphological and biochemical characteristics. Conventional tests were done including Gram stain, catalase test, indole production, methyl red test, urea hydrolysis, hydrogen sulphide production (TSI), citrate test, and fermentation of lactose, glucose, maltose, sucrose, and mannitol (Balows *et al*., 1991 and Elmer *et al*., 1997).

Bacterial identification by API 12A (12E) system (Oxoid)

The Microbact™ Gram-negative system was used for the identification of aerobic and facultatively anaerobic Gram-negative bacteria (Enterobacteriaceae and miscellaneous Gram- negative bacteria) according to (Bilkey *et al*., 1988).

Template DNA extraction according to (Srithan and Barker, 1991)

The DNA for PCR was obtained by suspending colonies of bacteria in 500 µl of sterile phosphate buffer saline (PBS), pH 8.0, then washing 3 times in PBS.

- The cell suspension was centrifuged for 10 min at14,000 xg.
- The supernatant was discarded carefully.
- The bacterial pellets were resuspended in 400 µl Tris-EDTA buffer (pH 8.0) and heated in heat block at 105^0C for 25 minutes, left to cool at room temperature and centrifuged at 14.000 xg for 10 minutes.
- The supernatant was transferred to a fresh tube with double volume 3M sodium acetate (pH 5.2) and the test tubes were kept at -20^0C for overnight.
- The DNA was pelleted by centrifugation at 14,000 xg/minute for 20 minutes, followed by washing with 70% ethanol and re-centrifugation at 14,000 xg/minute for 10 minutes.
- The DNA pellet was dried and resuspended in 20 µl sterile distilled water and stored at-20^0C till use.

Multiplex PCR

The procedure was originally derived from Stacy-Phipps *et al*. (1995) and Caeiro *et al*. (1999). Four primers were used to detect the genes encoding LT and ST in order to detect all types of ETEC in a single multiplex reaction. The oligonucleotides used to amplify a 450-bp segment of the LT-A gene were an upstream primer TW20 (5'GGCGACAGATTATACC GTGC-3'), nucleotides 4 to 23 in Yamamoto *et al*. (1987); primer LTPr1 described by Frankel *et al*. (1989) and a downstream primer JW11 (5'-CGGTCTCTATATTCCCTGTT-3'), nucleotides 443 to 424. The oligonucleotides used to amplify a 190-bp segment of the STI genes (estA1-4 alleles) were an upstream primer JW14 (5'-ATTTTTATTTCTGTATT GTCTT-3'), nucleotides 22 to 43 in Moseley *et al*.(1983), and a downstream primer JW7 (5'CACCCGGTACAGGCAGGATT 3'), nucleotides 212 to 193. The multiplex PCR was performed as follows: the 5µl of extracted DNA, 25µl of master mix, 1.5 Pmol of each four primers completed to 50µl with sterile distilled water. The thermal cycler was programmed to first heated to 50 ºC for 2 min and 95 ºC for 5 min and were then subjected to 40 cycles. Each cycle consisted of denaturation at 95 ºC for 45 s and annealing at 48 ºC for 45 s and extension at 72 ºC for 1 min and a final extension at 72 ºC for 10 minutes. After that the product was kept at 4ºC. The PCR products were separated by gel electrophoresis on agarose gel (1.5%) (Caeiro *et al*., 1999). One kb DNA molecular weight marker **(Qiagen)** was ran on gel, in addition to PCR products from LT positive and ST positive *E. coli* strains. The gel was stained with 0.5% ethidium bromide solution for 15 minutes and visualized using UV transilluminator.

Statistics

Statistical presentation and analysis of the present study was conducted, using the Chi-square tests by SPSS V17. Chi-square the hypothesis that the row and column variables are independent, without

indicating strength or direction of the relationship. Pearson chi-square and likelihood-ratio chi-square. Fisher's exact test and Yates' corrected chi-square are computed for 2x2 tables.

3. Results

One hundred and twenty five bacterial isolates were obtained from 135 children complained from diarrhea and 15 as healthy control. The obtained bacterial isolates were identified by Gram stain and Conventional biochemical tests into *E. coil* (87 isolates), *Klebsiella* spp (22 isolates), *Enterobacter* spp (3 isolates), *Serratia marcescens* (4 isolates) and non lactose fermentation species (9 isolates). Table 1 shows the different identified bacterial species in relation to the patients age, sex, fever and duration of diarrhea. In general, there were significant between age groups and the percentages of isolates from each species ($P > 0.05$). In this study, there were no significant effect for sex, fever, duration of diarrhea and isolated species from patients ($P< 0.05$).

API 12A system was done for 20 isolates which were identified by conventional biochemical reactions as *E. coli*. 18 out of 20 isolates were identified as *E. coli* (90%), one isolate as *Klebsiella oxytoca* and one isolate as *Serratia marcescens* (Table 2).

Fifty *E. coli* isolates identified by API 12A and conventional biochemical reactions were subjected for multiplex PCR for detection of LT and ST genes. Fig 1**,** the PCR products in gel (A) showed that, isolates no 4, 5 and 6 gave ST positive result with 190 bp, while isolate no 7 gave LT positive with 450 bp. On the other hand, positive PCR product for two genes (ST and LT) appeared in 9 and 11 isolates. In gel (B), the PCR products exhibited that, isolates no 3 gave LT positive with 450 bp, while isolates no 6, 9, 10 and 11 gave ST positive with 190 bp, and positive PCR product for two genes (ST and LT) appeared in 5 and 7 isolates. In gel (C), isolates no 7, 8 and 11 gave ST positive with 190 bp and positive PCR product for two genes (ST and LT) appeared in 6 isolate.

Out of the fifty (8 from them as control) *E. coli* isolates tested for presence of LT and/or ST genes, two isolates (4.0%) were positive for LT gene, ten isolates (20.0%) were positive for ST gene. LT and ST genes were detected in 5 isolates (10.0%). Neither LT or ST were detected in the rest of samples (33, 66.0%) (Fig. 2).

Table (3) shows only LT gene detected in 2 isolates from age group 3-10 years old, while detection of ST gene increased in the isolates (5 isolates, 50%) of age group 3-5 years old, then decreased in the isolates (3 isolates, 30%) of age group 6-10 years old and continue to decline for isolates (2 isolates, 20 %) of age group 11-15 years old. Accordingly ST and LT genes detection increased in the isolates (4 isolates, 80%) of age group 3-5 years old, then decreased in the isolates (1 isolate, 20%) of age group 6-10 years old and not appear in isolates of age 11-15 years old, the relation was not statistically significant between age and detection of each LT, ST and both ST/ LT. LT gene was found in (1/24) isolate of the male patients and in (1/26) isolate of the female patients, while ST gene was recorded in (6/24) isolates of the male patients and (1/26) isolate of the female patients, and LT/ST were found in (4/24) isolates of male patients and (1/26) isolate of female patients. The difference between them was not statistically significant ($P< 0.05$), and the relations were not statistically significant between fever, duration of diarrhea and detection of each LT, ST and both ST/LT.

As shown in Fig 3**,** 33 isolates were from Shebin El koum Teaching Hospital patients, positive ETEC was detected in 11/33 (33.3%). In El Hussein Hospital, positive ETEC was detected in 4/6 (66.6%). In Sayed Galal Hospital, positive ETEC was detected in 1/3 (33.3%). In Nasser Institute, positive ETEC was detected in 1/1 (100%). In private laboratories, no ETEC was detected in 7 *E. coli* isolates.

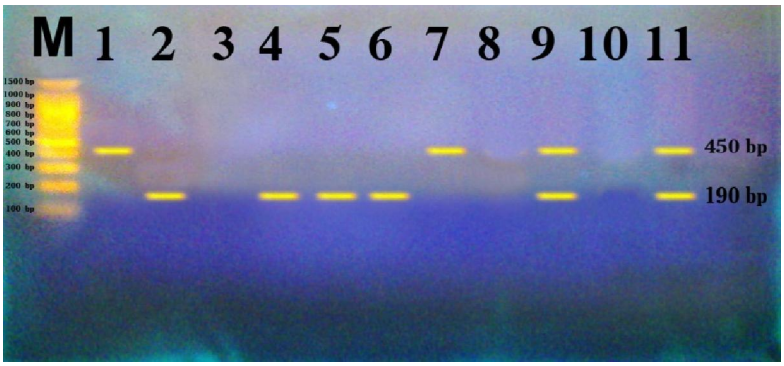
Table 1: Demographic characteristic of bacterial species isolated from diarrheic Egyptian children.

Characteristic		Bacterial species (Lactose and non lactose Fermentation)												Chi-Square	
		E.coli (n=87)		*Klebsiella* spp (n=22)		*Enterobacter* spp (n=3)		*Serratia marcescens* (n=4)		Non Lactose Fermentation species (n=9)		Total			
		N	%	N	%	N	%	N	%	N	%	N	%	χ^2	*P*-value
Age (year)	3-5.	57	65.52	8	36.36	3	100.00	4	100.00	9	100.00	81	64.80	23.516	0.003*
	6-10.	18	20.69	5	22.73	0	0.00	0	0.00	0	0.00	23	18.40		
	11-15.	12	13.79	9	40.91	0	0.00	0	0.00	0	0.00	21	16.80		
Sex	Male	50	57.47	14	63.64	2	66.67	2	50.00	4	44.44	72	57.60	1.162	0.884
	Female	37	42.53	8	36.36	1	33.33	2	50.00	5	55.56	53	42.40		
Fever	Positive	55	63.22	16	72.73	1	33.33	2	50.00	3	33.33	77	61.60	5.529	0.237
	Negative	32	36.78	6	27.27	2	66.67	2	50.00	6	66.67	48	38.40		
Duration of diarrhea (days)	2	36	41.38	8	36.36	0	0.00	1	25.00	5	55.56	50	40.00	20.441	0.431
	3	30	34.48	9	40.91	1	33.33	2	50.00	0	0.00	42	33.60		
	4	7	8.05	4	18.18	1	33.33	1	25.00	1	11.11	14	11.20		
	5	11	12.64	1	4.55	1	33.33	0	0.00	3	33.33	16	12.80		
	6	2	2.30	0	0.00	0	0.00	0	0.00	0	0.00	2	1.60		
	7	1	1.15	0	0.00	0	0.00	0	0.00	0	0.00	1	0.80		

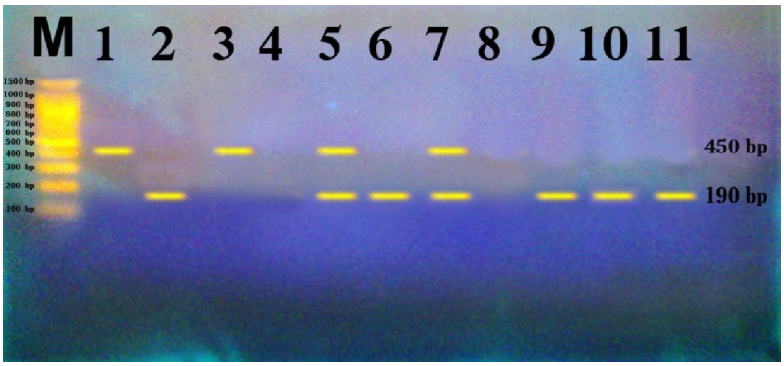
Table 2: Comparison between conventional biochemical reactions and API 12A system for identification of *E. coli*.

Bacterial species		Conventional biochemical reactions		API	
		N=20	%	N=20	%
E. coli		20	100.00	18	90.00
Not E. coli		*0*	*0.00*	2	10.00
Chi-Square	χ^2	2.105			
	P-value	0.349			

(A)



(B)



(C)

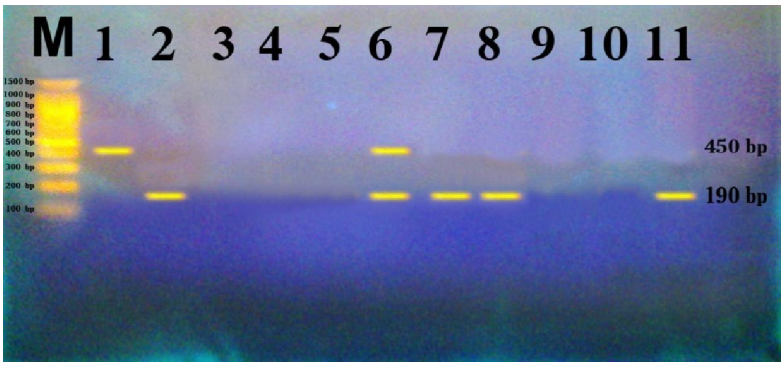


Fig 1: Gel electrophoresis of multiplex PCR products for detection of LT and ST genes in studied *E. coli* isolates. In gel A, B, C lane 1 as LT positive control around 450 bp and lane 2 as ST positive control around 190 bp. In gel (A), lane 3 as negative control, lane 4, 5, 6 as ST positive PCR products, lane 7 as LT positive PCR products and lane 9, 11 as ST&LT positive PCR product. In gel (B), lane 3 as LT positive PCR products, lane 5, 7 as ST&LT positive PCR product and lane 6, 9,10, 11 as ST positive PCR products. In gel (C), lane 6 as ST&LT positive PCR product and lane 7, 8, 11 as ST positive PCR products.

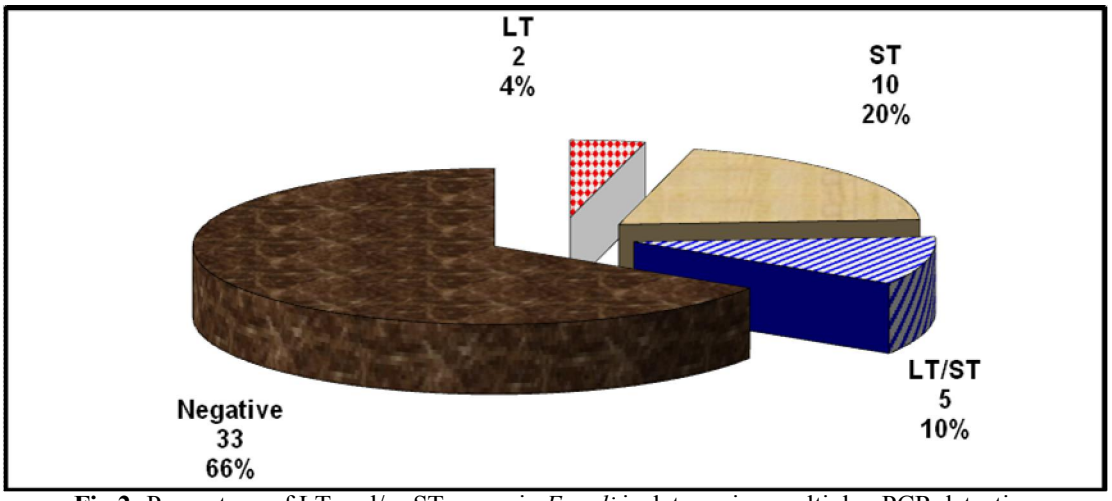


Fig 2: Percentage of LT and/or ST genes in *E. coli* isolates using multiplex PCR detection

Table 3: Demographic characteristic of Enterotoxigenic *E. coli* in Egyptian children with diarrhea.

Characteristics		PCR										Chi-Square	
		Negative (n=33)		LT (n=2)		ST (n=10)		LT/ST (n=5)		Total			
		N	%	N	%	N	%	N	%	N	%	χ^2	*P*-value
Age (year)	3-5.	25	75.76	1	50	5	50	4	80	35	70	4.429	0.618
	6-10.	5	15.15	1	50	3	30	1	20	10	20		
	11-15.	3	9.09	0	0	2	20	0	0	5	10		
Sex	Male	13	39.39	1	50	6	60	4	80	24	48	3.611	0.306
	Female	20	60.61	1	50	4	40	1	20	26	52		
Fever	Positive	22	66.67	2	100	8	80	4	80	36	72	1.72	0.632
	Negative	11	33.33	0	0	2	20	1	20	14	28		
Duration of diarrhea (days)	2	8	24.24	1	50	6	60	0	0	15	35.71	18.31	0.246
	3	9	27.27	0	0	2	20	3	60	14	33.33		
	4	5	15.15	0	0	1	10	2	40	8	16		
	5	6	18.18	0	0	1	10	0	0	7	16.67		
	6	3	9.09	1	50	0	0	0	0	4	8		
	7	2	6.06	0	0	0	0	0	0	2	4		

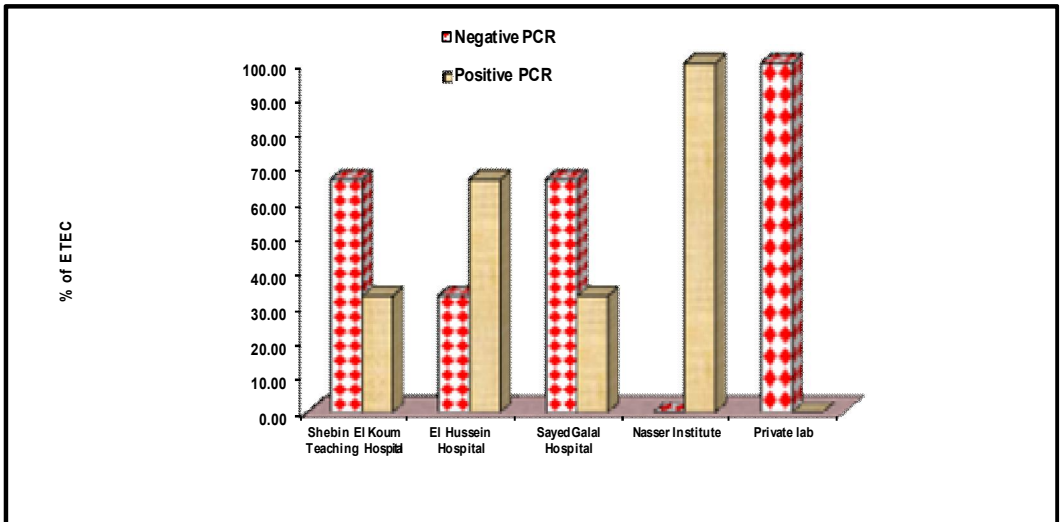


Fig 3: Incidence of LT and/or ST *E. coli* isolates in relation to different places.

4. Discussion

Acute diarrheal disease is an important health problem among children. Enterotoxigenic *Escherichia coli* is a common cause of childhood diarrhea in the developing world where sanitation and clean supplies of drinking water are inadequate. ETEC causes watery diarrhea, which can range from mild, self-limiting disease to severe-purging disease, generating severe dehydration that could lead to hospitalization and death (Huilan *et al*., 1991; Gonzales *et al*., 2013; Vilchez *et al.*, 2014).

Out of 125 bacterial isolates obtained in this study from diarrheagenic stool samples, 87 bacterial isolates (69.6%) were identified as *E. coli*. Manikandan *et al.* (2013) observed that 55.2% of incidence of *E. coli* was recorded. Our results showed that diarrheagenic *E. coli* was the major enteropathogenic bacteria in group aged (3-5) years (57/87, 65.5%). Bodhidatta *et al*. (2010)**,** reported that *E. coli* was commonly isolated from cases and controls in age under five years. Among 125 bacterial isolates, 72 isolates (57.6%) were isolates from males and 53 isolates (42.4%) were isolates from females, with no significant difference for sex group. This results is in agreement with Hossain *et al*. (2013)**,** who reported that, males had higher percentage of *E. coli* than females (64.2%, 35.8%, respectively).

90% of *E. coli* isolates that initially identified by conventional biochemical tests was identified as *E. coli* by API 12A. Roshdy *et al*. (2006) reported that, agreement between API system and conventional biochemical reactions was 78.4%. This indicates that the conventional biochemical reactions could be used only as a presumptive identification method. A disadvantage of conventional biochemical reactions is that they need more effort for preparations and interpretation and less accurate than recent methods but with comparatively lower cost.

In this study**,** two isolates were PCR positive for LT gene (2/17, 11.8%), ten isolates were PCR positive for ST gene (10/17, 58.8%) and five isolates were PCR positive for ST and LT gene (5/17, 29.4%). Different studies have shown differences in the predominant toxin type; for instance, LT ETEC was the most common toxin type in Bolivia (Gonzales *et al*., 2013)**,** LT/ST ETEC strains were found to be most prevalent in Peru (Rivera *et al*., 2010), ST-only ETEC strains in Brazil (Nunes *et al.*, 2011) and LT ETEC strains in Argentina (Viboud *et al*., 1999) and in Nicaragua (Vilchez *et al*., 2009). This was in agreement with (Bodhidatta *et al*., 2010), who reported that ST (48%), LT (17%) and ST and LT (35%), and disagreement with another studies carried out in Libya (Ali *et al*., 2012) and Mexico (Estrada-Garcia *et al*., 2009), where in Libya, all ETEC had only genes coding for ST, but in Mexico, heat- labile enterotoxin (LT) positive only. Only LT gene in this study was restricted to *E. coli* isolated from age groups 3-10 years, while ST gene was detected in all age groups but LT/ST were most common in age group 3-5 years and very low in age group 6-10 years (only one isolate). No significant relation has been found between age and the enterotoxigenic *E. coli.* ETEC was found to be the main etiological agent of diarrhea among children under five years of age (Iseri *et al*., 2011). Our results agree with Gomez-Duarte *et al.* (2013), they reported that children with diarrhea positive for ETEC included children from all ages.

E. coli isolates derived from male patients exhibited ST and ST/LT genes higher than female patients, but occurrence of LT gene was the same in *E. coli* isolates obtained from female and male patients, the difference between them was not statistically significant ($P< 0.05$). The incidence of ST and LT- ETEC for males were higher than that females (Rao *et al*., 2003). There was significant relation between sex distribution and ETEC (Gomez-Duarte *et al*., 2013). Most of patients in our study with LT- and ST- ETEC were suffered from fever that was observed in 72% of them, while 69% of Egyptian diarrhaeogenic children with ETEC were have diarrhea from 2-3 days. This is in agreement with study in Thailand (Bodhidatta *et al.*, 2010), who reported that, 69% from patients suffered from fever and the mean duration of diarrhea was 48.2 hours.

Our ETEC isolates were from Shebin El koum Teaching Hospital (33.3%), El Hussein Hospital (66.6%), Sayed Galal Hospital (33.3%), Nasser Institute (100%) and Private lab (0.0%). The incidence of the ETEC were recorded in most studied places. There were no significant between collection samples places and PCR detection of each LT, ST and ST/LT ($P > 0.05$). While (Germani *et al.*, 1994), who reported that, ETEC were significant greater for patients from urban area than for patients from rural areas.

Conclusions

This study has shown that Enterotoxigenic *E. coli* are major causes of acute diarrhea in children in Egypt especially in rural areas. The use of multiplex PCR system to detect enterotoxigenic *E. coli* with greater precision. We recommend extending the study to target more virulence genes in a large group of underline children with diarrhea. This can greatly facilitate the process of development of a vaccine against enterotoxigenic *E. coli* which should be considered a public health priority in Egypt as well as other developing countries.

References

1. Ali, M. M.; Mohamed, K. Z.; Klena, J. D.; Ahmed, S. F.; Moussa, T. A.; Ghenghesh, K. S. (2012). Molecular Characterization of Diarrheagenic *Escherichia coli* from Libya. Am J Trop Med Hyg 2012 vol. 86 no. 5 866-871.
2. Balows, A., Hausler, J. r. W. J.; Herrmann, K. L.; Isenberg, H. D.; Shadomy, H. J. (1991). Manual of Clinical Microbiology (Fifth edition). American Society for Microbiology, Washington. DC.
3. Bilkey, M. K.; Bremner, D. A.; Cameron, G. L.; Garner, J. G. (1988). Comparison of five commercial methods for the Identification of Non-fermentative and Oxidase Positive Fermentative Gram-Negative Bacilli. N.Z.J. Med. Lab Technol., 8-12.
4. Bodhidatta, L.; McDaniel, P.; Sornsakrin, S.; Srijan, A.; Serichantalergs, O. J.; Mason, C. (2010). Case-Control Study of Diarrheal Disease Etiology in a Remote Rural Area in Western Thailand. Am J Trop Med Hyg. Nov 5, 2010; 83(5): 1106–1109.
5. Caeiro, J-P., Estrada-Garcia, M. T.; Jiang, Z-D.; Mathewson, J. J.; Adachi, J. A.; Steffen, R.; DuPont, H.L. (1999). Improved detection of enterotogegenic *Escherichia coli* among patients with travelers' diarrhea, by use of the polymerase chain reaction technique. J. Infect. Dis. 180:2053-2055.
6. Clarke, S. C. (2001). Diarrhoeagenic *Escherichia coli*-an emerging problem? Diagn Microbiol Infect Dis 41: 93–98.
7. Elmer, W. K.; Stephen, D. A.; William, M. J.; Paul, C. S.; Washington, C. W. (1997). Color atlas and textbook of diagnostic microbiology, 5th edition. Lippincott-Raven Publishers, Philadelphia, New York.
8. Estrada-Garcia, T.; Catalina Lopez-Saucedo.; Rocio.; Thompson-Bonilla.; Maricela Abonce.; Daniel Lopez-Hernandez.; Jose Ignacio Santos.; Jorge L. Rosado.; Herbert, L.; DuPont.; Kurt, Z. Long. (2009). Association of Diarrheagenic *Escherichia coli* Pathotypes with Infection and Diarrhea among Mexican Children and Association of Atypical Enteropathogenic *E. coli* with Acute Diarrhea. J Clin Microbiol. Jan 2009; 47(1): 93–98.
9. Frankel, G., Giron, J.A., Valmassoi, J., Schoolnik. (1989). Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. Mol. Microbiol. 3: 1729-1734.
10. Germani, Y.; Morillon, M.; Begoud, E.; Dubourdiev, H.; Costa, R.; Thevenon, J. (1994). Two-Year Study of Endemic Enteric Pathogens Associated with Acute Diarrhea in New Caledonia. Journal of clinical microbiology, June 1994, p. 1532-1536 Vol. 32, No. 60095-1 137/94.
11. Gómez-Duarte, O. G.; Bai, J.; Newell, E. (2009). Detection of *Escherichia coli*, Salmonella spp., Shigella spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and Campylobacter spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagn Microbiol Infect Dis 63: 1-9.
12. Gomez-Duarte, O. G.; Romero-Herazo, Y. C., Paez-Canro, C.Z., Eslava-Schmalbach, J. H.; Arzuza, O. (2013). Enterotoxigenic *Escherichia coli* associated with childhood diarrhoea in Colombia, South America. J Infect Dev Ctries. 2013 May 13;7 (5):372-81. doi: 10.3855/jidc.2667.
13. Gonzales, L.; Sanchez, S.; Zambrana, S.; Iñiguez, V.; Wiklund, G.; Svennerholm, A. M.; Sjöling, A. (2013). Molecular Characterization of Enterotoxigenic *Escherichia coli* Isolates Recovered from Children with Diarrhea during a 4-Year Period (2007 to 2010) in Bolivia. J Clin Microbiol. Apr 2013; 51(4): 1219–1225.
14. Guerrant, R. L.; Kosek, M.; Moore, S.; Lorntz, B.; Brantley, R.; Lima, A.A. (2002). Magnitude and impact of diarrheal diseases. Arch Med Res 33: 351-355.
15. Hornes, E.; Wasteson, Y.; Olsvik, Ø. (1991). Detection of *Escherichia coli* heat-stable enterotoxin genes in pig stool specimens by an immobilized, colorimetric, nested polymerase chain reaction. J.Clin. Microbiol. 29: 2375-2379.
16. Hossain, Asif, M. D.; Raton, A. K.; Noor, R. (2013). Bacteriological study of stool samples collected from children suffering from diarrhea. Journal of Global Biosciences Vol. 2(5), 2013, pp. 160-165.
17. Huilan, S., Zhen, L.G., Mathan, M.M., Mathew, M.M., Olarte, J., Espejo, R., Khin Maun, U., Ghafoor, M.A., Khan, M.A., Sami, Z. (1991). Etiology of acute diarrhoea among children in developing countries: a multicentre study in five countries. Bull World Health Organ 69: 549-555.
18. Işeri, L.; Zafer Apan, T.; Aksoy, A.; Koç, F.; Sedef Göçmen, J.; Nuristani, D. (2011). The Prevalence of Enterotoxigenic *E. Coli* Isolated from the Stools of Children Aged 0-10 Years with Diarrhea in Mid-Anatolia Region, Turkey. Braz J Microbiol. 2011 Jan-Mar; 42(1): 243–247.
19. Kaper, J. B.; Nataro, J. P.; Mobley, H. L. (2004). Pathogenic *Escherichia coli*. Nat Rev Microbiol 2: 123-140.

20. Mamani, R.; Blanco, J.; Blanco, J. E.; Wiklund, G.; Svennerholm, A. M.; Sjoling, A.; Iniguez, V. (2011). Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhea in Bolivia. Braz. J. Infect. Dis. 15:132–137.
21. Manikandan, C.; Amsath, A.; Prabakaran, P. (2013). Random amplified polymorphic DNA-PCR analysis on bacterial strains from children diarrhea. Int.J.Curr. Microbiol. App. Sci (2013) 2(5): 306-314.
22. Moseley, S. L.; Hardy, J. W.; Hug, M. I.; Echeverria, P., Falkow, S. (1983). Isolation and nucleotide sequence determination of a gene encoding a heat stable enterotoxin of *Escherichia coli*. Infect Immun. 39: 1167-1174.
23. Mulcahy, N. (2013). Recommended drug may cause diarrhea with radiation. Medscape Medical News. Available http://www.medscape.com/viewarticle/812057. Accessed October 7, 2013.
24. Nataro, J. P. and Kaper, J. B. (1998). Diarrhoeagenic *Escherichia coli*. Clin. Microbiol. Rev., 11: 142-201.
25. Nunes, M. R.; Penna, F. J.; Franco, R. T.; Mendes, E. N.; Magalhaes, P. P. (2011). Enterotoxigenic *Escherichia coli* in children with acute diarrhea and controls in Teresina/PI, Brazil: distribution of enterotoxin and colonization factor genes. J. Appl. Microbiol. 111:224–232.
26. Persson, S.; de Boer, R. F.; Kooistra-Smid, A. M. D.; Olsen, K.E.P. (2011). Five commercial DNA extraction systems tested and compared on a stool sample collection. Diagn Microbiol Infect Dis 69: 240-244.
27. Rao, M. R.; Abu-Elyazeed, R.; Savarino, S. J.; Naficy, A. B.; Wierzba, T. F.; Abdel-mMassih, I.; Shaheen, H.; Frenk, R. W.; Svennerholm, A. M.; Clemens, J. D. (2003). High disease burden of diarrhea due to enterotoxigenic *Escherichia coli* among rural Egyptian infants and young children. J. Clin. Microbiol. 41: 4862-4864.
28. Rivera, F. P.; Ochoa. T. J.; Maves, R. C.; Bernal, M.; Medina, A. M.; Meza, R.; Barletta, F.; Mercado, E.; Ecker, L.; Gil, A. I.; Hall, E. R.; Huicho, L.; Lanata, C. F. (2010). Genotypic and phenotypic characterization of enterotoxigenic Escherichia coli strains isolated from Peruvian children. J. Clin. Microbiol. 48:3198–3203.
29. Roshdy, R. A.; El-Essawy, A. K.; Abu Shady, H. M.; Ibrahim, M. R.; Abu shady, M. R. (2006). Validity of multiplex PCR as an emerging technique for diagnosis of enterotoxigenic *Escherichia coli*. Egypt. J. Med. Lab. Sci., (ESIC), Vol. 15, No. 1, March 2006.
30. Roy, S.; Shamsuzzaman, S. M.; Kazi, Z. M. (2014). Molecular Detection of Diarrheagenic Escherichia coli from Children with Acute Diarrhea in Tertiary Care Hospitals of Dhaka, Bangladesh. Asian Journal of Medical Science, Volume-5(2) 2014: 59-66.
31. Sritharan, V. and Barker, R. H. (1991). "A simple method for diagnosing *M. tuberculosis* infection in clinical samples using PCR" Mol. Cell. Probes 5:385-395 (1991).
32. Stacy-Phipps, S.; Mecca, J. J.; Weiss, J. B. (1995). Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during course of infection. J Clin Microbiol 33: 1054-1059.
33. Thielman, N. M. and Guerrant, R. L. (2004). Clinical practice. Acute infectious diarrhea. N Engl J Med 350: 38-47.
34. Viboud, G. I.; Jouve, M. J.; Binszten, N.; Vergara, M.; Rivas, M.; Quiroga, M.; Svennerholm, A. M. (1999). Prospective Cohort Study of Enterotoxigenic *Escherichia coli* Infections in Argentinean Children. J. Clin. Microbiol. vol. 37 no. 9 2829-2833.
35. Vilchez, S.; Reyes, D.; Paniagua, M.; Bucardo, F.; Mollby, R.; Weintraub, A. (2009). Prevalence of diarrhoeagenic Escherichia coli in children from Leon, Nicaragua. J. Med. Microbiol. 58:630–637.
36. Vilchez, S.; Becker-Dreps, S.; Amaya, E.; Perez, C.; Paniagua, M.; Reyes, D.; Espinoza, F.; Weintraub, A. (2014). Characterization of enterotoxigenic *Escherichia coli* strains isolated from Nicaraguan children in hospital, primary care and immunity setting. J Med Microbiol 2014 May 19;63 (Pt 5):729-34.
37. Yamamoto, T.; Gojobori, T.; Yokota, T. (1987). Evolutionary origin of pathogenic determinants in entero-toxigenic *Escherichia coli* and *Vibrio cholerae* O1. J. Bacteriol. 169: 1352-1357.

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