Egyptian Journal of Medical Microbiology Volume 25 / No.3 / July 2016 59-65

ORIGINAL ARTICLE

Diarrheagenic *Escherichia Coli* (DEC): Detection by Multiplex PCR and Antibiotic Susceptibility Pattern

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Key words:

Diarrhea; Diarrheagenic E coli; Antibiotic susceptibility

ABSTRACT

Background*: Diarrheagenic Escherichia coli (DEC) are among the most important causes of diarrheal diseases in children.* ***Objectives:*** *This study aimed* ***to*** *detect the virulence loci associated with 4 categories of DEC among Egyptian children with and without diarrhea, and to get an idea about their antibiotic resistance pattern.* ***Methodology:*** *E coli were isolated from 104 diarrheal fecal samples and 104 non-diarrhea fecal samples. Multiplex PCR was performed to characterize target genes of DEC. Drug susceptibility patterns were determined by disc- diffusion method.* ***Results:*** *The target genes identifying DEC were detected in 21 diarrheal specimens (20.2%) including genes for ETEC (8 specimens, 7.7%); EPEC (6 specimens, 5.8%); EAEC (6 specimens, 5.8%) and one strain (1%) positive for genes of both ETEC and EPEC. DEC genes were detected in 3 out of 104 non-diarrheal specimens (2.9%); ETEC, 1% and EAEC, 2% with high significant difference between diarrheal and non-diarrheal groups. Antimicrobial susceptibility testing revealed high frequency of resistance of two groups to ampicillin, tetracycline and trimethoprim-sulphamethoxale (76.2% were multidrug resistant) ,low frequency of resistance to ampicillin-sulbactam, ceftazidim, ceftriaxone and azetronam and no resistance to cefepime, imipenem, amikacin or gentamicin.* ***Conclusion****: DEC is a major pathogen in childhood diarrhea in Egypt and the strains being susceptible to multiple antibiotics, although a large proportion (76.2%) of them were multi- drug resistant*

INTRODUCTION

Diarrhea is a common cause of morbidity and mortality among infants and young children in the developing countries[1]**.** The causes of diarrhea include many viruses, bacteria and parasites. Diarrheagenic *Escherichia coli* (DEC) is an important bacterial cause of endemic and epidemic diarrhea worldwide[2].

Diarrheagenic *Escherichia coli* strains can be classified into six main types according to their serotypes ,the presence of specific virulence properties and specific epidemiological and clinical features: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Enterohemorrhagic *E. coli* (EHEC) ,and Diffusely adherent *E. coli* (DAEC)[3]**.**

Enteropathogenic *E. coli* causes a histopathological lesion known as attaching and effacing (A/E). Strains of the A/E genotype, which do not possess the gene of bundle forming pili (bfpA) are classified as atypical EPEC (a-EPEC). The term Shiga toxin (Stx) -producing *E. coli* (STEC) is now used to define any *E. coli* strain that produces Stx, and EHEC is used to denote the STEC strains that also contain locus of enterocyte effacement [4]**.**

Enterotoxigenic *E. coli* is associated with watery diarrhea among children in developing countries and its pathogenesis is due to production of heat-labile (lt), heat-stable (st) or both toxins. EAEC is defined as *E. coli* strains that adhere in vitro to HEp-2cells in autoaggregative (AA) pattern. EIEC are capable of invading colonic epithelial cells, followed by dysentery. DAEC has a characteristic diffuse pattern of adhesion to HEp-2 cells[5]**.**

Cultural and biochemical criteria can't be used alone for identification of DEC, since they can't differentiate them from the non-pathogenic *E. coli* commonly found in human feces. Moreover, specific serotyping is not usually correlated with pathogenicity. Since several virulence factors and DNA sequences of DEC have been identified, DEC can be detected by the presence of genes coding for these specific virulence factors, which are not present in non-pathogenic strains[6].

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Various multiplex PCR combining specific primer pairs for DEC pathotypes virulence genes have been developed to detect different types of DEC in a single-step reaction. These methods showed high sensitivity and high specificity for identification of DEC[6,7]. We use the multiplex PCR as a rapid diagnostic tool for detection of ETEC, EPEC, EHEC and EAEC by multiplex PCR, using primers specific to virulence genes in order to determine the prevalence of these 4 categories of DEC among children with and without diarrhea; and then get an idea about their antibiotic susceptibility pattern.

METHDOLOGY

1- Study Area and Population

During the period from March 2013 and April 2014, stool samples were collected from children with (cases) and without diarrhea (controls) attending the pediatric outpatient clinic of Zagazig University Hospitals. They were from 2-10 years old. Clinical and epidemiological information were obtained through questionnaires. The study was approved after receiving permission from Zagazig University Ethical Committee and consent from children's parents. Control subjects were healthy children with no history of diarrhea for at least one month attending the clinic for non- diarrheal illness. Neither cases nor controls had been treated with antibiotics in the 2 weeks preceding sampling. The samples were investigated in Microbiology and immunology Department, Faculty of Medicine, Zagazig University to determine the prevalence of DEC as a part of a case control study.

2- Pathogen Identification

After microscopic examination for helminthes and protozoa and exclusion of stool specimens with gross infestation with parasites, the study was conducted on 104 stool samples of diarrhea and 104 stool samples without diarrhea. Stool samples from all children were cultured on MacConkey agar and Sorbitol MacConkey agar (Oxoid, UK) then incubated aerobically at 37 for 24 hours. About 5 lactose fermenting colonies and 5 sorbitol non-fermenting colonies (if present) presumed to be *E. coli* by colony morphology and confirmed by APi 20E and biochemical tests were selected and stored in Trypticase soy broth supplemented with 20% glycerol at - 80°C.

3- Reference Strains

Reference strains (*E.coli* ATCC 43887 positive for attaching and effacing (eaeA) and bfpA genes of EPEC; *E.coli* ATCC 35401 positive for lt and st genes of ETEC; EAEC 042 positive for AA gene of EAEC; and O157:H7 positive for stx1, stx2 and eaeA genes of EHEC were used as positive controls of the multiplex PCR assay. The positive strains were cultured on MacConkey agar at 37 °C. A sweep of about five *E.coli* colonies was used for PCR[8].

The DNA was isolated from colonies as described[9]**.** Having confirmed the specificity of each primer (Table 1) by monoplex PCR, the DNA templates were subjected to multiplex PCR as described below

4- Multiplex PCR for Detection of Virulence Genes of DEC Strains Isolated From Stool Samples

Preserved *E coli* samples of cases and controls were subcultured on MacConkey plates. After incubation at 37°C overnight, a smear from the first area of the plate was taken for DNA extraction as described[9], the extracted DNA was stored at -20°C until being used for DNA amplification. The DNA templates were subjected to multiplex PCR using primers (Table 1) as described[6,7]**.** Alkaline phosphatase house-keeping gene (PhoA) was amplified in all samples [10]. All primers were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Each sample was examined in 2 multiplex PCR reactions using Maxime PCR PreMix Kit (iNtRON Biotechnology, Korea).

a. Multiplex PCR assay 1

Two µl of the DNA template and 5 pmol/µl (1 µl) of forward and reverse primers for LT, ST genes of ETEC and phoA internal control gene, 20 pmol/µl (1 µl) of forward and reverse primers of AA gene of EAEC were added to each bead in 20 µl mixture .

b. Multiplex PCR assay 2

Two µl of the DNA template, 5 pmol/µl (1 µl) of each forward and reverse primers of genes(bfp, eaeA for EPEC and stx1, stx2 for EHEC and phoA) were added to each bead in 20 µl mixture .

For the negative control reaction, all components of PCR reaction were added to the bead except for DNA. The gene segments were amplified using DNA thermal cycler (**Biometra, UK**). The PCR cycles for both PCR reactions were carried out as follows: 94°C for 5 minute, followed by 40 cycles (each, 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 1minutes), and a final extension at 72°C for 10 minutes.

PCR products were analyzed by gel electrophoresis with 2.0% (W/V) agarose gel (**Boehringer Mannheim, Germany**) in Tris acetate EDTA (TAE) (1X) buffer. DNA Molecular weight marker ranging from 100 to 1000 bp (iNtRON Biotechnology, Korea) was loaded in each run. The DNA bands were visualized and photographed under UV light after staining the gel with ethidium bromide (Sigma, USA). Specimens that revealed DEC were subjected to monoplex PCR for more conformation of the test and of mixed infection**.**

Table I: Primers used in the multiplex PCR for amplification of DEC genes.

E. coli ***category***	***Locus***	***Primers***	***Amplicon size (bp)***
ETEC	*lt*	F:5´-GGC GAC AGA TTA TAC CGT GC-3´ R:5´- CGG TCT CTA TAT TCC CTG TT-3´	440
ETEC	st	F:5´-ATT TTT CTT TCT GTA TTG TCT T-3´ R:5´-CAC CCG GTA CAA GCA GGA TT-3´	191
EPEC	bfpA	F:5´-AAT GGT GCT TGC GCT TGC TGC-3´ R:5´- GCC GCT TTA TCC AAC CTG GTA-3´	324
EPEC	eaeA	F:5´ -GAC CCG GCA CAA GCA TAA GC-3´ R:5´-CCA CCT GCA GCA ACA AGA GG-3´	384
STEC	stx1	F:5´-CTG GAT TTA ATG TCG CAT AGT G-3´ R:5´-AGA ACG CCC ACT GAG ATC ATC-3´	150
STEC	stx2	F:5´-GGC ACT GTC TGA AAC TGC TCC-3´ R:5´-TCG CCA GTT ATC TGA CAT TCT G-3´	255
EAEC	AA	F : 5´-CTGGCGAAAGACTGTATCAT-3´ R:5´-CAATGT ATAGAAATCCGCTGTT-3´	630
E. coli	PhoA	Pho-F GTGACAAAAGCCCGGACACCATAAATGCCT Pho-R TACACTGTCATTACGTTGCGGATTTGGCGT	900

ETEC, enterotoxigenic *E.coli*; EPEC, enteropathogenic *E.coli*; STEC, Shiga-toxin–producing *E.coli*; EAEC, enteroaggregative*E.coli*. *lt, heat-labile toxin;* st, heat-stable toxin; bfpA , bundle-forming pilus; eaeA, attaching and effacing; stx1 ,shiga toxin 1; stx2, shiga toxin 2; AA, aggregative adherence; PhoA, alkaline ohosphatase house-keeping gene.

5- Antibiotic Susceptibility Test of *E. Coli* Isolates

Antibiotic susceptibility of 21 DEC of case group and 30 of *E. coli* normal flora of control group was done by the disc- diffusion method on Muller- Hinton agar (**Becton-Dickinson, USA**) and the growth inhibition zones were interpreted according to the National Committee for Clinical Laboratory Standards guidelines[11]with the intermediate and resistant categories were grouped together as non-susceptible . The antimicrobial discs included Ampicillin, Ampicillin-sulbactam, Ceftazidime, Ceftriaxone, Cefepime, Imipenem, Amikacin, Gentamicin, Tetracycline, Aztreonam and Trimethoprim-sulphamethoxazole (**Oxoid, UK**). Multi-resistance was defined as non-susceptibility to at least three families of antibiotics.

6- Statistical Analysis

The chi-square test (X^2) and Fisher exact test were used to determine the statistical significance of the data. *P* value of <0.05 was considered significant.

RESULTS

Multiplex PCR for Reference Strains

Multiplex PCR assays showed 100% specificity in identifying the reference strains. Nonspecific bands were not visualized.

Multiplex PCR for Detection of DEC Strains Isolated from Stool Samples

The multiplex PCR detected targeted genes of DEC (Fig. 1) in 21 out of 104 (20.2%) diarrheal samples and in 3 out of 104 (2.9%) non-diarrheal stool samples. The prevalence of DEC in both groups was significantly different (*p*<0.001). The main DEC identified in both groups and the significant association with diarrhea were ETEC, EPEC and EAEC (table 2). The prevalence was greatest for ETEC among children with diarrhea. ETEC and atypical EPEC were detected in 1 specimen denoting mixed infection. Out of the 8 ETEC strains isolated, 4(50%) produced lt only and 2 (25%) st only and remaining 2 (25%) produced both toxins. 5 of the 6 EPEC (83.33%) isolated from children with diarrhea were atypical EPEC (positive for eaeA gene only) and one isolate (16.66%) was typical EPEC (positive for both eaeA and bfpA genes). The prevalence of DEC was higher among male (16; 76.2%) than in female (5; 23.8%) and in children aged 2-5 years (18; 85.7%) than in older children (3; 14.3%) and in rural area (15; 71.4%) than in urban area (6; 28.6%) with statistically significant differences.The result of antibiotic susceptibility testing of *E. coli* (DEC and normal flora) are shown in table 3.The high frequency of resistance of both DEC and normal flora were observed to ampicillin, tetracycline and trimethoprim-sulphamethoxale, with 16/18 strains (76.2%) being multi-drug resistant to these 3 antibiotics. Low frequency of resistance to ampicillin-sulbactam, ceftazidim, ceftriaxone and azetronam and no resistance to cefepime, imipenem, amikacin and gentamicin. There were statistically non-significant differences in antibiotic resistance between DEC of diarrheal children and normal flora of control group.

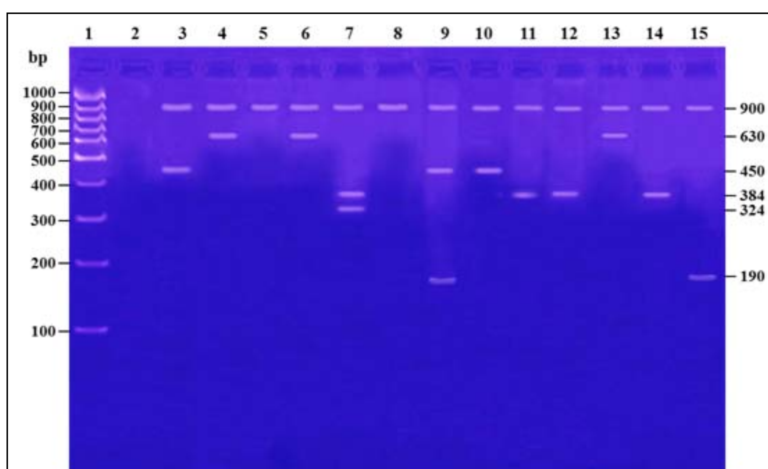

Fig. 1: Agarose gel showing results of multiplex PCR of clinical sample*s*.

Lane1: Molecular size marker (100-1000 bp). **Lane 2:** Negative control (no bands). **Lane 3:** ETEC (LT amplicon, size 440bp and PhoA amplicon, size 900pb); **Lane 4, 6,13:** EAEC (AA amplicon, size 630 bp and PhoA amplicon),**Lane 5, 8:** non DEC (PhoA amplicon, size 900pb)**. Lane7**: tEPEC (eaeA& bfpA amplicons , sizes 384, 324 bp and PhoA internal control amplicon ,size 900) . **Lane 9:** ETEC (LT& ST amplicons, size 440, 190 bp and PhoA amplicon). **Lane10,11:**Represent PCR sample which was positive for both ETEC& aEPEC where **lane 10** was loaded with first multiplex PCR (LT amplicon, size 440 & PhoA amplicon, size 900 pb) and **lane 11** loaded with the second multiplex PCR (eaeA amplicon , sizes 384 of aEPEC& PhoA amplicon, size 900 pb). **Lane 12,14 :** aEPEC (eaeA amplicon , sizes 384 of & PhoA amplicon, size 900 pb) **Lane 15 :** ETEC (ST amplicons, size 190 bp and PhoA amplicon).

Table 2:Diarrheagenic *E.coli* among children with and without diarrhea.

	With diarrhea (NO=104)		***Without diarrhea (NO=104)***		X^2	***p.value***
	NO.	**%**	**NO.**	**%**		
ETEC	8	7.7	1	1.0	10.9	<0.001 **
EPEC	6	5.8	0	0.0	12.0	<0.001 **
EHEC	0	0.0	0	0.0		
EAEC	6	5.8	2	2.0	4.0	0.046 *
+Mixed infection	1	1.0	0	0.0	2.0	0.157

X^2 (chi-square test).

* P < 0.05: significant **P< 0.001: highly significant +Mixed infection of ETEC and atypical EPEC.

ETEC, enterotoxoigenic*E.coli*; EPEC,enteropathogenic*E.coli*.

EHEC, enterohaemorrhagic*E.coli*; EAEC,enteroaggregative*E.coli.*

Table 3: Antibiotic resistance of DEC of diarrheal group and normal flora of control group.

	DEC of diarrheal group No.=21		***Normal flora of control group No.=101***		***p.value***
	NO.	**%**	**NO.**	**%**	
Ampicillin	18	85.7	92	91.1	0.451
Ampicillin- sulbactam	6	28.6	24	23.8	0.641
ceftazidim	4	19.0	16	15.8	0.718
Ceftriaxone	4	19.0	18	17.8	0.894
cefepime	0	0.0	0	0.0	
Imipenem	0	0.0	0	0.0	
Amikacin	0	0.0	0	0.0	
Gentamicin	0	0.0	0	0.0	
Tetracycline	16	76.2	72	71.3	0.648
azetronam	2	9.5	9	8.9	0.928
Trimethoprim- sulphamethoxazole	16	76.2	66	65.3	0.335

X^2 (chi-square test*)* used for comparison between both groups. *P* > 0.05 : non significant

DISCUSSION

Diarrheagenic *E. coli* has been identified as an important cause of infantile and young childhood diarrhea in all the developing countries where it has been looked for, but the incidence has varied greatly in different studies. In this study, DEC strains were isolated from 20.2 % of cases compared to 2.9% of controls which is more or less similar to a previous study in Zagazig city (24.4%, 3.3%) [12] however, it was higher than another Egyptian study (12.5% and 3%) [13]. The geographical distribution areas, socioeconomic status and the difference in the age groups are responsible for different results as the later study was made on infants live in Alexandria city. Reports from Iran [14] and Brazil [6] are in accordance with our results. However reports from Arabian countries are different as DEC **was** 70.4% in Tunisia[15], 8.6% in Libyen children[16] and 38% in Iraq[17] **.**

The most prevalent pathotype isolated was ETEC (7.7%) that is agrees with many studies done in Egypt[12,13,18] and in Tunisia[15]. On the other hand, in Brazil the prevalence of ETEC was 3.7%, 2.9% and 2.7% predominated by EAEC and EPEC[6,19,20].

EHEC were not isolated from both groups of children. The absence of EHEC as a cause of acute diarrhea in children in the present study was in agreement with reports from Egypt[12,13] and other countries[14,17,21,22]. However, EHEC were reported in 10.4% of diarrheal children in Tunisia [15] and in 0.5%., 7.4% and 0.2% in Brazil[6,19,20]. The difference of food consumption habits especially consumption of uncooked meat, presence of fast food chain restaurants, probably account in part for difference in transmission of EHEC from animals to humans.

Enteroaggregative *E. coli and EPEC* was equally present in our cases (5.8% each) with the exception that EAEC was also detected in the stool of non- diarrheal children (2.0%) with significant difference. The EAEC has been implicated as a cause of diarrhea in developing countries and gastroenteritis outbreaks in some industrialized countries[20]. This result approximately similar to those reported in Libya, India and Brazil[16,23,24], whereas other investigators reported high rates (20–38%) for EAEC from children with diarrhea in Brazil and Tanzania[20,25]**.**

Genes for atypical EPEC and ETEC (eae<) were detected simultaneously in one specimen accounting for 1% of cases. There were also reports of different co-infection of DEC strains as by EPEC and ETEC[13], three different combinations of DEC strains (ETEC/EPEC, ,ETEC/EIEC and ETEC/EHEC)[15] and 2 different combinations (a-EPEC/EAEC, EAEC /ETEC) [6].

This study showed the predominance of lt producing ETEC (50%) compared to st producing ETEC (25%) and both lt and st producing one (25%). This result is more or less similar to that reported previously[12,13,18,26]**.** Contrasting with this result, ST producing ETEC were predominant in other reports[14,17,27]**.** The disagreement between these results may due to loss of plasmid carrying the gene of one of both toxins.

Five of the 6 EPEC were eae PCR positive and bfpA negative (a- EPEC) and one was eae and bfpA PCR positive (typical EPEC). bfpA is the structural gene encoding BFP (the bundle-forming pilus). This gene is expressed only under certain culture conditions[28]. This could be the reason for low frequency of typical EPEC in our study, as shown also in other studies[12,13,16,20,29]. Moreover, the decrease in the occurrence of typical EPEC and the increase in the atypical EPEC as a cause of diarrhea can be related to the fact that typical EPEC is almost exclusively detected in humans, whereas atypical EPEC can also be detected in domestic animals, which may behave as reservoir for these bacteria [30]**.**

Analysis of data showed that children who revealed DEC were significantly from rural area, as was reported previously in Egypt[18,31] and in other countries[16,20] due to poor general living conditions, low level of education and bad personal hygiene. The incidence of DEC was higher in males than females. The same finding has been reported previously[13,17,32] as in rural areas, boys spend most of time outside playing in street and eat Junk food. DEC was significantly associated with children less than 5 years of age (85.7%) than in older children (14.3%) as was reported from many studies in developing countries[29,33]**,** this because defenses are frequently deficient or lacking in the infant and young children.

Treatment of childhood diarrhea by using antibiotics is limited due to WHO recommendations against routine antibiotics for uncomplicated secretory diarrhea[34]**.** Yet, knowledge about antibiotic sensitivity pattern of DEC may be of value for treatment of severe and complicated cases[35]. It may also be valuable for prophylaxis and treatment of traveler's diarrhea caused by ETEC[36].

Antibiotic susceptibility of DEC of case group and normal flora *E. coli* of control group were tested by disc diffusion method. High level of resistance to ampicillin, while low level of resistance to the combination of ampicillin with beta-lactamase inhibitor sulbactam suggests the production of beta-lactamase as a mechanism of resistance in *E. coli* including DEC. Low level of resistance to ceftazidime and ceftriaxone, while no resistance to fourth generation cephalosporins cefepime in DEC and normal flora may indicate that the isolated strains of *E.coli* were low level extended-spectrum beta-lactamase-producers. The similarity of resistance in DEC and normal flora *E. coli* imply that the strains were likely to have originated from the community, which supports the observation of low levels of resistance to these antibiotics[37].

A large proportion (76.2%, 65.3%) of DEC and normal flora *E. coli* respectively were multi- drug resistant (MDR), showing resistance to ampicillin, trimethoprim-sulphamethoxazole and tetracycline. Multidrug resistant *E coli* has been reported in many studies in Egypt[13,38] and different parts of the world[19,29,39]. Long-term use of these classes of antibiotics has been suggested to be responsible for the high level of resistance against them[40] .

All the strains were susceptible to imipenem, amikacin and gentamicin .In accordance with our result, many studies [13,19,39] recorded lack of resistance of *E. coli* to these antibiotics. Based on this observation, these antibiotics could be very useful as drugs of choice for therapeutic purposes against severe cases of *E. coli* in hospitals.

In conclusion, this study has shown that DEC is a major pathogen in childhood diarrhea in Egypt particularly in rural areas, although their incidence has been declined. The use of multiplex PCR system can distinguish the different categories of DEC with great precision. DEC still sensitive to many antibiotics which can be used in severe complicated cases although a large proportion of them were multi- drug MDR, showing resistance to ampicillin, trimethoprim-sulphamethoxazole and tetracycline.. We recommended extending the study to target more virulence genes in a large group of children with diarrhea to identify other pathotypesof DEC.

REFERENCES

1. Bryce J, Boschi-Pinto C, Shibuya K, Black RE , the WHO Child Health Epidemiology Reference Group: WHO estimates of the causes of death in children. Lancet 2005; 365: 1147-1152.
2. Nweze EI: Virulence properties of diarrheagenic *E. coli* and etiology of diarrhea in infants, young children and other age groups in Southeast, Nigeria. Am Euras J Sci Res. 2009; 4: 173-9.
3. Aranda KR, Fagundes-Neto U, Scaletsky IC: Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic *Escherichia coli* and *Shigella* spp. J Clin Microbiol. 2004; 42: 5849-53.
4. Kaper JB, Nataro JP, Mobley HLT: Pathogenic *Escherichia coli.* Nat Rev Microbiol. 2004;2:123-40.
5. Jafari A, Aslani MM, Bouzari S: *Escherichia coli*: a brief review of iarrheagenic pathotypes and their role in diarrheal diseases in Iran. Iran J Microbiol. 2012; 4(3): 102–117.
6. Bueris V, Sircili M P, Taddei C R, Fernandes dos Santos M, Franzolin MR, Martinez MB, et al: Detection of diarrheagenic *Escherichia coli* from children with and without diarrhea in Salvador, Brazil. Mem Inst Oswaldo Cruz. 2007; 2(7): 839-844.
7. Lopez-Saucedo C, Cerna JF, Villegas-Sepulveda N, Thompson R, Velazquez F R, Torres J, et al: Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. Emerg Infect Dis. 2003; 9(1):127-31.
8. Hedge A, Ballal M, Shenoy S: Detection of diarrheagenic *Escherichia coli* by multiplex PCR. Indian J Med Microbiol. 2012; 30:279-284.
9. Boom R, Sol C J, Salimans M M, Jansen C L, Vander J: Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990; 28: 495-503.
10. Kong RYC, So CL, Law W F, Wu RSS: A sensitive and versatile multiplex PCR system for the rapid detection of enterotoxigenic (ETEC), enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) strains of *Escherichia coli*. Mar Pollut Bull. 1990; 38(12): 1207-1215.
11. Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility, 22nd Informational Supplement. M100-S22, CLSI Wayne PA. 2012.
12. Allam AA, Amer A, Fahmy K, Siam AG: Rapid diagnosis and characterization of diarrheagenic *Escherichia coli* in Egyptian children using multiplex PCR. Egypt. J Med Microbiol. 2006; 15(3): 523.
13. El Metwally HAR, Ibrahim HAH, El-Athamna MN, Amer MA : Multiplex PCR for detection of diarrheagenic *Escherichia coli* in Egyptian children. J Med Sci. 2007; 7(2):255-262.
14. Alikhani MY, Hashemi SH, Aslani MM, Farajinia S: Prevalence and antibiotic resistance patterns of diarrheagenic *Escherichia coli* isolated from adolescents and adults in Hamedan, Western Iran. Iranian J Microbiol. 2013; 5(1):42-47.
15. Al-Gallas N, Bahri O, Bouratbeen A, Haasen AB, Aissa RB: Etiology of acute diarrhea in children and adults in Tunis, Tunisia, with emphasis on diarrheagenic *Escherichia coli*: Prevalence, phenotyping, and molecular epidemiology. Am J Trop Med Hyg. 2007; 77(3): 571-582.
16. Ali MM, Mohamed ZK, Klena JD, Ahmed SF, Moussa TAA, Ghenghesh KS: Molecular characterization of diarrheagenic *Escherichia coli* form Libya. Am J Trop Med Hyg. 2012; 86(5): 866-871.
17. Arif SK and Salih LIF: Identification of different categories of diarrheagenic *Escherichia coli* in stool samples by using multiplex PCR technique. Asian J Med Sci 2010; 2(5): 237-243.
18. Mansour A, Shaheen HI, Amine M, Hassan K, Sanders JW, Riddle MS, et al: Pathogenicity and phenotypic characterization fo enterotoxigenic *Escherichia coli* isolates from a birth cohort of

children in rural Egypt. J Clin Microbiol. 2014; 52(2): 587.
19. Garcia PG, Silva VL, Diniz CG: Occurrence and antimicrobial drug susceptibility patterns of commensal and diarrheagenic *Escherichia coli* in fecal microbiota from children with and without acute diarrhea. J Microbiol.2011; 49(1): 46-52.
20. Lozer DM, Souza T B, Monfardini MV: Genotypic and phenotypic analysis of diarrheagenic *Escherichia coli* strains isolated from Brazilian children living in low socioeconomic level communities. BMC Infect Dis. 2013; 13: 418.
21. Güney C, Aydogan H, Saracli MA, Basustaoglu A, Doganci L: No isolation of *Escherichia coli* O157:H7 strains from faecal specimens of Turkish children with acute gastroenteritis. J. Health Popul. Nutr. 2001; 19(4): 336-337.
22. Ghenghesh KS, Franka EA, Tawil K A, Abeid S, Ali MB, Taher I A, et al: Infectious acute diarrhea in Libyan children: causative agents, clinical features, treatment and prevention. Libyan J Infect Dis. 2008; 2(1): 10-19.
23. Nair GB, Ramamurthy T, Bhattacharya MK, Krishnan T, Ganguly S, Saha DR, et al: Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrheal patients in Kolkata, India. Gut Pathog. 2010; 2: 4.
24. Orlandi PP, Magalha˜es G F, Matos BN, Silva T, Penatti M, Nogueira PA, et al: Etiology of diarrheal infections in children of Porto Velho (Rondonia, western Amazon region, Brazil). Braz J Med Biol Res. 2006; 39: 507–517.
25. Vargas M, Gascon J, Casals C: Etiology of diarrhea in children less than five years of age in Ifakara, Tanzania. Am J Trop Med Hyg. 2004; 70: 536–539..
26. Guerra JA, Romero-Herazo YC, Arzuza O, Gomez-Duarte OG: Phenotypic and genotypic characterization of enterotoxigenic *Eschierichia coli* clinical isolates form Northern Colombia, South America. Biomed Res Int. 2014; Article ID 236260.
27. El-Mohamady H, Ibrahim A, Fouad G: Enteric pathogens associated with diarrhea in children in Fayoum, Egypt. Diag Microbiol Infect Dis. 2006; 56: 1-5.
28. Nataro J P, Kaper JB: Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998; 11(1): 142-201.
29. Nguyen TV, Le VP, Le CH, Weintraub A: Detection and characterization of diarrheagenic Escherichia coli from young children in Hanoi, Vietnam. J Clin Microbiol. 2005; 43:755-60.
30. Paula CJ S and Marin JM: Isolation of extraintestinal pathogenic Escherichia coli from diarrheic dogs and their antimicrobial resistance profile. Braz J Microbiol. .2008; 39: 498-500.
31. Oyofo BA, Peruski LF, Ismail TF, El-Etr SH, Churilla A M, Wasfy MO, et al: Enteropathogens associated with diarrhea among military personnel during Operation Bright Star 96, in Alexandria, Egypt. Mil Med. 1997; 162:396-400.
32. Moyo SJ, Maselle SY, Matee MI, Matee M, Langel N, Mylvaganam H: Identification of diarrheagenic Escherichia coli isolated from infants and children in Dar es Salaam, Tanzania. BMC Infect Dis. 2007; 7: 92.
33. Gautam K, Pokhrel BM, shrestha CD, Bhatta DR: Prevalence of Diarrheagenic *E.coli* (DEC) determined by polymerase chain reaction (PCR) in different tertiary care hospitals in Nepal. JSM Microbiol. 2015; 3(2): 1021 .
34. World Health Organization: The treatment of diarrhea: a manual for physicians and other senior health workers (4**th** rev). W.H.O., Geneva, Switzerland. 2005.
35. Pickering LK and Snyder JD. Gastroenteritis. In: Behrman, RE, Kliegman RM, Jenson HB, eds. Nelson Textbook of Pediatrics. 2**0th**ed. Philadelphia: WB Saunders Company, 2004, pp765-767.
36. Ericsson CD: Traveller's diarrhoea. Int J Antimicrob Agents. 2003; 21: 116-124.
37. Dropa M, Balsalobre LC, Lincopan N, Mamizuka E M, Murakami T, Cassettari V C, et al: Extended-spectrum beta-lactamase among Enterobacteriaceae isolated in a Public hospital in Brazil. Rev. Inst. Med. Trop. Sao Paulo. 2009; 51: 203-209.
38. Zakaria AM, Abdel Aziz M H, Selim SA: Multi-drug resistant (MDR) E. Coli origenated from clinical and environmental sources in Ismailia, Egypt. EJARBLS. 2015; 3(1):8-20.
39. Adefisoye M A and Okoh AI: Identification and antimicrobial resistance prevalence of pathogenic Escherichia coli strains from treated waste water effluents in Eastern Cape, South Africa. Microbiol Open. 2016; 5:143-151.
40. Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ, et al: Antimicrobial drug resistance in Escherichia coli from humans and food animals, United States, 1950–2002. Emerg Infect Dis 2012;18(5) 18:5.