Isolation and identification of different diarrheagenic (DEC) Escherichia coli pathotypes from children under five years old in Baghdad

Zena K. Khalil

Abstract

Background: Diarrheagenic *Escherichia coli* strains can be classified into six main pathotypes on the basis of their specific virulence properties, association with some serotypes, and different epidemiological and clinical features. The identification of DEC cannot be based only on cultural and biochemical criteria, since they are indistinguishable from the non-pathogenic *E. coli* and specific serotyping is not always correlated with pathogenicity. Since several virulence factors and DNA sequences of DEC have been identified, they can be determined by the presence of

Materials and Methods: From June 2014 –September 2014 , one hundred thirty two stool samples were collected from diarrheal children admitted to the Pediatric Central Hospital in Baghdad and different diarrheagenic (DEC) *Escherichia coli* pathotypes isolated by using multiplex polymerase chain reaction , the susceptibility of isolated *Escherichia coli* to some selected antibiotics were identified by using disc diffusion method . In order to distinguish typical and atypical EPEC strains, these were tested for the presence of EAF plasmid.

Results: The frequency of diarrheagenic *E. coli* was (38.6%) and the most frequently isolated pathotypes was EAEC (35.3%), followed by atypical EPEC (29.4%), ETEC (21.6%),and STEC (7.8%). EPEC+ STEC (3.9%) and typical EPEC was detected only in one sample (2%). The frequency of the pathotypes studied were (89%) occurred among children less than 2 years old. EAEC was mainly detected among children under the age of 1 year (61%) , EPEC in children aged younger than 2 years (87%) and ETEC in children older than 1 year (64%) . Among children with diarrhea who were positive for DEC, (80.4%) had watery diarrhea and (19.6%) had bloody diarrhea. Fever was the most commonly symptom reported in association with DEC (76.5%) followed by vomiting (19.5%) and dehydration (4%), and the highest sensitivity *E. coli* isolates was recorded for Cefotaxime (92%) followed by Ciprofloxacin (90%) and Gentamicin (82%), while the highest resistance was observed against Oxytetracycline (84%) and Doxycycline hydrochloride (80%) followed by Ampicillin (77%) and Co-trimoxazole (75%) .

Conclusions: This study reveals that DEC strains contribute to the burden of children diarrheal diseases and EAEC is the most commonly identified DEC strain. The highest sensitivity *E. coli* isolates was recorded for Cefotaxime (92%) .

Keywords: Children, Baghdad city, diarrheagenic *Escherichia coli*

Introduction :

Diarrheal disease is a major public health problem throughout the world, with over two million deaths occurring each year, affecting mostly children under 5 years of age in developing countries . [1,2].

The etiological agents include a wide range of viruses, bacteria and parasites. Among bacterial pathogens, diarrhoeagenic *Escherichia coli* (DEC) are important agents of endemic and epidemic diarrhea worldwide [3].

The DEC can be divided into several groups based on their distinct virulence determinants: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC) being the most important [4]. .

Multiplex PCR allows fast and accurate detection of more than one virulence factor in a single PCR reaction, and it is a relatively new method that can simultaneously amplify template mixture and decrease the detection costs, conquering the weakness of single PCR detecting only one template once [5, 6] .

The aim of the study was to identify different diarrheagenic (DEC) *Escherichia coli* pathotypes isolated from 132 children with acute diarrhea under 5 years old and to determine the susceptibility of *Escherichia coli* isolates to some selective antibiotics.

Patients and methods :

During the period from June 2014 to September 2014, samples were collected from 132 outpatient children under five years age who had diarrhea defined as three or more loose, liquid, or watery stools or at least one bloody loose stool in 24 h , admitted at The Pediatrics Central Hospital in Baghdad city .

Information's about age , types of diarrhea and clinical symptoms (fever, vomiting and dehydration) were recorded for each diarrheal child using a questionnaire. Samples were taken by using a swab transport system and transported to the Laboratory within 2 h of collection.

The samples were inoculated into Mac-Conkey broth for enrichment at 37°C for 24 h, the enrichments were streaked on Mac-Conkey agar and incubated for 24 h at 37ºC. Pink, colored colonies isolates were sub cultured on Eosin Methelene Blue (EMB) agar. Colonies producing greenish metallic sheen on EMB agar were considered as having *E. coli*. In addition, various biochemical tests were done for the confirmation of *E. coli* . (7) .

E. coli isolates were stored at -80°C in trypticase soy broth (TSB) supplemented with 20% glycerol for further procedures .

Multiplex PCR : The M-PCR was done in the laboratories of the Iraqi Biotechnology Co. in Baghdad.

DNA was extracted from selected pure cultures of bacteria that had been enriched at 37c° for 18 h in trypticase soy broth ,one ml of the broth was collected in an eppendorf tube, centrifuged at 15000 rpm for 5 min and the supernatant discarded, the cell pellets were resuspended in 500 µl of sterile deionized water and boiled for 10 min. The multiplex PCR was developed by combining five specific primer pairs for EPEC, STEC/ EHEC, ETEC, and EAEC virulence genes, as described in table (1) . Each multiplex reaction was performed in a 50 µl final volume containing 1 µl of the template DNA, 0.2 mM DNTPs, 10 mM Tris-HCl (pH8.8), 1.5 mM MgCl2, 50 mM KCl, 2 U Taq DNA polymerase (Invitrogen), and 10 pmol of each primer (Biosynthesis). DNA templates from the stored *E.coli* isolates were submitted to multiplex PCR

The thermo cycling conditions were as follows: an initial denaturation step of 94°C for 5 min, 94°C for 45 min,50°C for 1 min, and 2 min and 72°C for 35 cycles, with a final elongation step of 9 min extension at 72°C. Amplified samples were evaluated by 1% agarose gel electrophoresis in Tris-borate- EDTA buffer(Pronadisa, Madrid, Spain) and stained with 2 µg of ethidium bromide (Sigma-Aldrich, Madrid, Spain) per ml and photographed under UV light .

DEC detection :

The *eae*-positive strains were tested by PCR for the presence of EAF plasmid, as described by (8). In all experiments, the prototype EPEC E2348/69, EAEC 042, ETEC H10407 (LT), ETEC C275/15 (ST), and EHEC O157:H7 strains were used as positive control, while *E. coli* K12 DH5α was the negative control.

Antibiotics sensitivity test:

The antibiotic susceptibility tests were performed as per method described by (9) to find out the antibiotic sensitivity of *E. coli* In vitro antibiotic sensitivity test of the *E. coli* isolates was conducted by paper disc diffusion method using the discs supplied by HiMedia Laboratories Pvt. Ltd., Mumbai (India). Antibiotics used in this test are Kanamycin (30µg), Ampicillin (10µg), Streptomycin (10µg), Amikacin (30µg), Cefotaxime (30µg), Oxytetracycline (30µg), Trimithoprime (5µg), Doxycycline Hydochloride (30µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Nalidixic Acid (30µg), Gentamicin (10µg), Levofloxacin 5µg) and Co-trimoxazole (20µg).

Statistical analysis :

The chi-square test was used to determine the statistical significance of the

data. A value of $p < 0.05$ indicated statistical significance.

Table 1 :Sequence of PCR primers, sizes of amplified DNA fragments and concentration of multiplex PCR

Gene	Sequence (5'- 3')	ProductSize bp	[pmol]	Reference
eae (EPEC)	IntFC-CCGGAATTCGGGATCGATTACCGTCAT IntRC- CCAAGCTTTTATTTATCAGCCTTAATCTC	820	100	(10)
LT 696 20 (ETEC)	LTFW- GGCGACAGATTATACCGTGC LTREV- CCGAATTCTGTTATATATGTC	696	20	(11)
ST (ETEC)	STFW - TCCCTCAGGATGCTAAAC STREV - GCAACAGGTACATACGTT	240	20	(12)
Stx (STEC)	StxUFW - GAACGAAATAATTTATATGT StxUREV - TTTGATTGTTACAGTCAT	500	75	(13)
AA (EAEC)	pCVD432FW- CTGGCGAAAGACTGTATCAT pCVD432REV-CAATGTATAGAAATCCGCTGTT	630	20	(14)

EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxigenic *E. coli*; STEC: Shiga toxin-producing *E. coli*; EAEC: Enteroaggregative *E. coli*.

Results:

A total of 132 children with diarrhea were studied. Diarrheagenic *E. coli* was isolated from 38.6% of the children with diarrhea (51 patients) .The frequency of each DEC pathotype is shown in table (2).

The most frequently isolated was EAEC (35.3%), followed by atypical EPEC (29.4%), ETEC (21.6%), and STEC (7.8%). Co-infections (EPEC+ ETEC) (3.9%) and typical EPEC was detected only in one sample (2%), and it belonged to serotype O142:H34.Seven of ETEC strains were positive for the LT gene and three for the ST gene, while one strains possessed both genes encoded by *est* and *elt* genes, respectively.

And three STEC strains that had *eae* gene identified by PCR belonged to serotypes O157:H7, O26:H11 and O111:H-, and no isolates of EIEC were detected ,as shown in figure (1) .

Table (2)
Frequency of *Escherichia coli* pathotypes in children with diarrhea

E. coli pathotype	Case patients (n = 51) (38.6%)
EAEC	18(35.3%)
EPEC	15(29.4 %)
ETEC	11(21.6 %)
STEC	4(7.8%)
Co-i EPEC+ ETEC	2(3.9%)
t-EPEC	1(2%)
Total	51 (100%)

Co-i , Co-infection , t- EPEC, typical EPEC

Considering the different age groups, DEC pathogroups were detected in all of them there was no significant differences of frequency of DEC and age groups , but most cases (89%) occurred among children <2 years old. EAEC was mainly detected among children under the age of 1 year (61%) , EPEC in children aged younger than 2 years (87%) and ETEC in. children older than 1 year (64%) . Among children with diarrhea who were positive for DEC, 41/51 (80.4%) had watery diarrhea and 10/51 (19.6%) had bloody diarrhea. Bloody diarrhea was more common in the EAEC than in the infections with the other DEC pathogroups .

Fever was the symptom most commonly reported in association with DEC, diarrhea (76.5%), followed by vomiting (19.5%) and dehydration (4%) as shown in table (3).

Table(3)
The relation between age variables and clinical characteristic with DEC pathogroups

Age variables and clinical characteristic	DEC Pathogroups						
	EAEC n=18	EPEC n=15	ETEC n=11	STEC n=4	Co-infection n=2	t-EPEC n=1	Total 51
Age in months 1-12	11(61%)	9(60%)	3 (27%)	0	0	0	23(45.1%)
13-24	5(28%)	4(27%)	7(64%)	1(25%)	1(50%)	0	18(35.3%)
25-59	2(11%)	2(13%)	1(9%)	3(75%)	1(50%)	1(100%)	10(19.6%)
Diarrhea types watery	14(78%)	12(80%)	9(82%)	3(75%)	2(100%)	1(100%)	41(80.4%)
bloody	4(22%)	3(20%)	2(18%)	1(25%)	0	o	10(19.6%)
Symptoms fever	16(89%)	10(66%)	7(64%)	3(75%)	2(100%)	1(100%)	39(76.5%)
vomiting	2(11%)	4(27%)	3(27%	1(25%)	0	1(100%)	10(19.5%)
dehydration	0	1(7%)	1(9%)	0	0	0	2(4%)

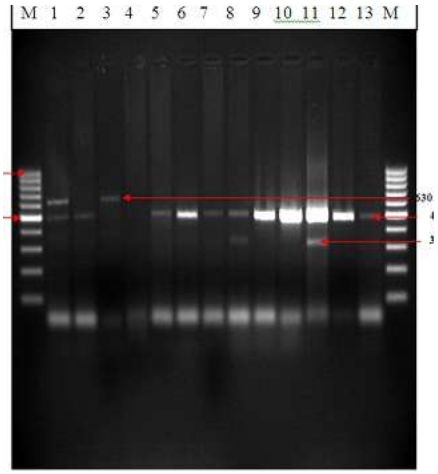

Figure (1)

Multiplex PCR results of some clinical samples

M: 100bp DNA ladder; Lane1: Sample (No. 3) (*eae* 482 bp and PCVD 630bp), Lane 2: Sample (No.10) (*eae* 482bp), Lane 3: Sample (No. 15) (PCVD 630bp), Lane 4: Sample (No. 2)(negative), Lane 5,6,7: Samples (No. 19),(No.1) and (No.13) (*eae* 482bp), Lane 8:Sample (No. 20) (LT450bp), Lane 9 and 10: Samples (No. 6) and (No.18) (ST160bp), Lane 11: Sample (No. 33) (*est* 482bp and 32 *elt*4bp), Lane 12 and 13: Sample (No. 31) and Sample (No.43). (ST160bp).

Antibiotic sensitivity test :

In present investigation among 51 *E. coli* isolates diarrheal children the highest sensitivity was recorded for Cefotaxime (92%) followed by Ciprofloxacin (90%) and Gentamicin (82%) . The highest resistance was observed against Oxytetracycline (84%) and Doxycycline hydochloride (80%) followed by Ampicillin (77%) and Co-trimoxazole (75%),as shown in table(4).

Table (4)

The antimicrobial sensitivity and resistance of *E. coli* isolates

Antimicrobial agents	*E. coli* n=51		
	Sensitive	intermediate	Resistant
Cefotaxime	47(92%)	2(4%)	2(4%)
Ciprofloxacin	46(90%)	1(2%)	4(8%)
Gentamicin	42(82%)	1(2%)	8(16%)
Trimithoprime	36(71%)	2(4%)	13(25.5%)
Nalidixic Acid	32(63%)	1(2%)	18(35%)
Levofloxacin	28(55%)	1(2%)	22(43%)
Streptomycin	22(43%)	2(4%)	27(53%)
Amikacin	20(39%)	1(2%)	30(59%)
Chloramphenicol	19(37%)	1(2%)	31(61%)
Kanamycin	13(25.5%)	2(4%)	36(71%)
Co-trimoxazole	10(20%)	3(6%)	38(75%)
Ampicillin	9(18%)	3(6%)	39(77%)
Deoxycycline hydrochloride	7(14%)	3(6%)	41(80%)
Oxytetracycline	6(12%)	2(4%)	43(84%)

Discussion :

Diarheagenic *E .coli* has been identified as an important cause of childhood diarrhea in all the developing countries ,but the incidence has varied greatly in different studies . The prevalence of DEC was significantly higher in children with diarrhea (38.6%) . Similar results have also been reported in southeastern and western Africa, like Mozambique and Nigeria . (15,16) . In Jordan DEC was (30%) in 2003. In our study, EAEC was the most commonly detected DEC pathogroup in children (35.3%) , making it the most prevalent type of diarrheagenic *E. coli* and this was agreed with other recent studies from other Southeast Asian countries including India, Thailand and Vietnam. (17,18,19). EPEC was second highest frequently isolated (29.4 %)whereas the frequency of t-EPEC was very low (2%) and this was agreed with other studies in Egypt and Kuwait . (20). And this showing a drastic decline in the frequency of typical EPEC with the frequency of atypical EPEC strains on the rise.

Up to the 1990s, typical EPEC was the main cause of acute diarrhea in children younger than one year old, of low socio-economic status in developed countries.. (21).

It is likely that the frequency of t- EPEC has been influenced by the recently improved public health measures, such as more efficient control of hospital infections and implementation of sanitary conditions..(22).

The prevalence of ETEC was (21.6%) which agreed with other previous studies, as the prevalence of ETEC was shown to be 20.7% in patients in Argentina and Nicaragua .(23). While other investigators have seen the low prevalence of ETEC in children with diarrhea in Iran.(24). The prevalence of STEC was (7.8%)These data are similar to those reported in a previous study in Egypt (7.6%) . (25) . In previous studies STEC have been identified in sporadic cases of non-bloody diarrhea, particularly in young children .(26,27) . In other study isolated three O157:H7 strains from bloody diarrhea, and one case due to an O26:H11 strain has been recently described in Brazil . (28). Among non-O157 STEC, serotypes O111:NM, O111:H8, and O26:H11 have been identified as agents of infantile diarrhea in Hungarian children . (29).

As another report studied eighteen strains of the O157:H7serotype isolated from bloody diarrhea in humans during different surveys conducted in Brazil, suggesting that this serotype is emerging in this country, These findings point to the need for more effective measures from our public health services.(30).

The frequency of the DEC pathogroups with age and clinical features that were examined, DEC were observed in children of all ages under 5 with an increased occurrence in children aged 1–12 months and those 13–24 months , as observed also in other study in Tunisia. (31).

Bloody diarrhea was less common than watery diarrhea probably because the number of patients with STEC, which are commonly associated with bloody diarrhea, was low. Also the other clinical symptoms were, in general, similar regardless of the detected DEC pathogroup, as has been reported in other studies from Mombasa and Bangladesh.(32).

In the present study most DEC strains isolated were resistant to Oxytetracycline (88 %) and Deoxycyclin (86%). Similar results reported in other studies indicate the universal resistance of DEC to these agents. Higher rates of resistance have been reported both in children and adults in recent years.(33,34).

In the current study, some of the DEC isolates were multidrug resistant (MDR). The most resistance pattern was to Oxytetracycline/ Deoxycyclin/Ampicillin . The incidence of diarrhea due to MDR *E. coli* has increased in developing countries in the last decade. Higher rates have been reported from 50 to 70% in recent years ,and the highest rate up to 75% have been reported from India. (35,36).

Inappropriate use of antibiotics may be responsible for the antimicrobial resistance. Also, dissemination of transferable plasmids encoding multidrug resistance has been observed in the DEC isolates from diarrheal stools and surface waters. Use of surface waters for drinking or irrigation raises an important hazard for dissemination of MDR DEC in the developing countries. (37). In the present study high resistance was reported to Kanamycin (74.5 %), In contrast to other study which showed that *E. coli* 157isolates were susceptible to Kanamycin . (38).

Conclusions:

This study reveals that DEC strains contribute to the burden of children diarrheal diseases and EAEC is the most commonly identified DEC strain.

Recommendations:

The indiscriminate use of antibiotics needs to be avoided and the guidelines for proper use of antibiotics for the treatment of diarrhea in many regions need to be established. and further studies are needed to investigate the ecological, socio-economic and epidemiological basis of this pathotype

References:

1. Khan R, Rizvi M, Shukia I, Malik A: A novel approach for identification of members of Enterobacteriaceae isolated from clinical samples, Biol Med 2011; 3:313.
2. Bryce J, Boschi-Pinto C, Shibuya K, Back RE. WHO estimates of the causes of death in children. Lancet 2005; 365: 1147–1152.
3. Madigan MT, Martinko JM, Stahl DA and Clark DP . Brock Biology of Microorganisms, Pearson Education, Inc., Permissions Department, 1900 E. Lake Ave., Glenview, United States of America,2011; 13th ed.
4. Bauman R. Machunis-Masuoka E. Microbiology With Diseases by Taxonomy, Pearson Education, Inc., Permissions Department, 1900 E. Lake Ave., Glenview, United States of America ,2014; 4th ed.
5. Welinder-Olsson C, Kaijser B. Enterohemorrhagic Escherichia coli (EHEC). Scand J Infect Dis 2005; 37: 405–416
6. Qadri F, Svennerholm AM, Faruque AS, Sack RB. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 2005; 18:465–483.
7. Nataro JP. Enteroaggregative Escherichia coli pathogenesis. Curr Opin Gastroenterol 2005;21: 4-8.
8 . Welinder-Olsson C, Kaijser B. Enterohemorrhagic Escherichia coli (EHEC). Scand J Infect Dis 2005; 37: 405–416 .
9. Nguyen T.V., Le Van P., Le Huy C.,Weintraub A. Antibiotic resistance in Diarrheagenic *Escherichia coli* and *Shigella* strains isolated from children in Hanoi, Vietnam. Antimicrobial Agents Chemother 2005; 49**:** 816–9.
10. Batchelor M, Knutton S, Caprioli A, Huter V, Zanial M, Dougan G, Frankel G. Development of a universal intimin antiserum and PCR primers. J Clin Microbiol 2009; *37*: 3822-387.
11. . 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 2006; 32: 2393-2397.
12. .Moseley SL, Hardy JW, Hug MI, Echeverria P, Falkow S.Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli*. Infect Immun 2008; *39*: 1167-1174.
13. Yamasaki S, Lin Z, Shirai H, Terai A, Oku Y, Ito H, Ohmura M,Karasawa T, Tsukamoto T, Kurazono H, Takeda Y.Typing of verotoxins by DNA colony hybridization with polyand oligonucleotide probes, a bead-enzyme-linked immunosorbent assay, and polymerase chain reaction. Microbiol Immunol 2009; 40: 345-52.
14. Schimidt H, Knop C, Franke S, Aleksic S, Heesemann J, Karch H. Development of PCR for screening of enteroaggregative *Escherichia coli*. J Clin Microbiol 2010;33:701-705.
15. Yamamoto T., Yokota T. Cloning of deoxyribonucleic acid regions encoding a heat-labile and heat-stable enterotoxin originating from an enterotoxigenic *Escherichia coli* strain of human origin. J Bacteriol 2006; 143**:** 652–60.
16. Okamoto K., Fujii Y., Akashi N., Hitotsubashi S., Kurazono H.,Karasawa T., Takeda, Y. Identification and characterizationof heat-stable enterotoxin II-producing *Escherichia coli* from patients with diarrhea. Microbiol Immunol 2005; 37**:** 411–4.
17 . Fujii Y., Okamuro Y., Hitotsubashi S., Saito A., Akashi N., Okamoto K. Effect of alterations of basic amino acid residues of *Escherichia coli* heat-stable enterotoxin II on enterotoxicity. Infect.Immun. 2006; 62**:** 2295–301.
18. Perelle, S., Dilasser, F., Grout, J. and Fach, P. Detection of Escherichia coli serogroup O103 by real-time polymerase chain reaction. J Appl Microbiol 2005 ; 98, 1162–1168.
19. Perelle, S., Dilasser, F., Grout, J. and Fach, P. Screening food raw materials for the presence of the world's most frequent clinical cases of Shiga toxin-encoding Escherichia
coli O26, O103, O111, O145 and O157. Int J Food Microbiol 2007; 113, 284–288.
20. Drysdale, M., MacRae, M., Strachan, N.J., Reid, T.M. and Ogden, I.D. The detection of non-O157 E. coli in food by immunomagnetic separation. J Appl Microbiol 2006 ; 999,223–226.
21. Afset JE, Bergh K, Bevanger L. High prevalence of atypical enteropathogenic *Escherichia coli* (EPEC) in Norwegian children with diarrhoea. J Med Microbiol 2005; *52*: 1015-1019.
22. Aranda KR, Fagundes-Neto U, Scaletsky IC. Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic of *Escherichia coli* and *Shigella* spp. J Clin. Microbio. 2006;*42*: 849-5853.
23. Barreto ML, Milroy CA, Strina A, Prado MS, Leite JP, Ramos EA, Ribeiro H, Alcantara-Neves NM, Teixeira MD, Rodrigues LC, Ruf H, Guerreiro H, Trabulsi LR. Community-based monitoring of diarrhea in urban Brazilian children:incidence and associated pathogens. Trans R Soc Trop Med Hyg 2006; 100: 234-242.
24. Alikhani, M.Y., A. Mirsalehian and M.M. Aslani,.Detection of typical and atypical enteropathogenic*Escherichia coli* (EPEC) in

Iranian children with and without Diarrhea. J. Med. Microbiol., 2006;55: 1159-1163.

25. Alikhani MY, Masoumi Asl H, Khairkhah M, Farajnia S, Aslani MM. Phenotypic and genotypic characterization of *Escherichia Coli* O111serotypes. Gastroenterol Hepatol From Bed to Bench. 2011;4:147–152.
26. Bastos FC, Vaz TM, Irino K, Guth BE. Phenotypic characteristics,virulence profile and genetic relatedness of O157 Shiga toxin-producing *Escherichia coli* isolated in Brazil and other Latin American countries. *FEMS* Microbiol. Lett 2006; 265: 89-97.
27. Cantarelli V, Nagayama K, Takahashi A, Honda T, Cauduro PF,Dias GAG, Mezzari A, Brodt T. Isolation of Shiga toxinproducing *Escherichia coli* (STEC) serotype O91:H21 from a child with diarrhea in Porto Alegre city, RS, Brazil. Braz. JMicrobiol. 2007; 31: 266-270.
28. Irino K, Vaz TMI, Kato MAMF, Naves ZVF, Lara RR, Marco MEC, Rocha MM, Moreira TP, Gomes TA, Guth BE. O157:H7 Shiga toxin-producing *Escherichia coli* strains associated with sporadic cases of diarrhea in São Paulo, Brazil.Emerg Infect Dis 2006; 8: 446-447.
29. Guth BEC, Vaz TM, TA Gomes TA, Chinarelli SH, Rocha MM,de Castro AF, Irino K. Re-emergence of O103:H2 Shiga toxin-producing *Escherichia coli* infections in São Paulo,

Brazil. J Med Microbiol2005; *54*: 805-806.

30. Bueris, V., M .P. Sircili, C.R. Taddei, M.F. Santos, M.R. Franzolin, M .B. Martinez, R. Ferrer, M.L. Barreto and L.R. Trabulsi,. Detection of diarrheagenic *Escherichia coli* from children with and without diarrhea in salvador, Bahia, Brazil. Mem Inst Oswaldo Cruz, Rio de Janeiro, 2007; 102(7): 839-844.
31. Kausar, Y., Chunchanur, S. K., Nadagir, S. D., Halesh, L. H. 21. LeJeune J. T. and Rajala-Schultz P. J. Food safety: and Chandrasekhar, M. R. virulence factor Serotypes and Antimicrobial Suspectibility Pattern of Escherichia coli in Urinary Tract Infections. Al Ameen J. Med. Sci. 2009; 2(1):47-51.
32. Albert, M.J., S.M. Faruque, A.S. Faruque, P.K. Neogi,M. Ansaruzzaman, N.A. Bhuiyan, K. Alam and M.S. Akbar. Controlled study of *Escherichiacoli* diarrheal infections in Bangladeshi children. J. Clin. Microbiol., 2005; 33(4): 973-977.
33. .Onono, J. O., Kangethe, E. K. and Ogara, W. O. Antimicrobial susceptibility of non-sorbitol fermenting Escherichia coli isolated from feaces *samples* African J. Microbiol. Res. 2010; 4(16): 1703-1707.
34. Browne R, Lior H, Prado V, Kaper JB, Nataro JP, Maneval D, Elsayed A, Levine MM. Characterization of enteroadherent-aggregative Escherichia coli, a putative agent of diarrheal disease. J Infect Dis. 2008 Jul;158(1):70–79 .
35. Scotland SM, Smith HR, Said B, Willshaw GA, Cheasty T, Rowe B. Identification of enteropathogenic Escherichia coli isolated in Britain as enteroaggregative or as members of a subclass of attaching-and-effacing E. coli not hybridising with the EPEC adherence-factor probe. J Med Microbiol. 2006 Nov;35(5):278–283.
36. Thaker, H. C., Brahmbhatt, M. N. and Nayak, J. B. Study on occurrence and antibiogram pattern of Escherichia coli from fecal samples in Anand, Gujarat, India. Vet. world. 2012; 5(9): 556-559.
37. Aslani MM, Alikhani MY. Molecular and phenotypic characterization of atypical enteropathogenic *Escherichia coli* serotypes isolated from children with and without diarrhea. J Microbiol Immunol Infect. 2011;44:27–32
38. Black JG . Microbiology: Principles and Explorations,2012; 8th ed. John Wiley &Sons, Ltd.

.