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Detection of *Escherichia coli* Pathotypes from the Cases of Diarrhea

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***Escherichia coli* is a member of the normal microbiota, and also a common inhabitant of the human and warm-blooded animal intestinal tract, which several pathogenic types of it can cause different diseases. One hundred and fourty seven *E. coli* isolates were obtained from the cases of diarrhea during 2013-2014. In order to detect *E. coli* pathotypes a mixture of nine primer pairs was used in three multiplex PCR assays. The PCR assays detected 26 ETEC, 20 EAEC, 11 EHEC, 9 EPEC and 2 EIEC. All *E. coli* strains were serogrouped by a latex agglutination test using a commercial antiserum. According to the results, 53 isolates were positive and according to kit regents, 3 different groups were identified. The findings also indicated that the *E. coli* strains recovered in this study expressed high levels of resistance to antimicrobials that are commonly used in clinical medicine. In conclusion, differentiation between the diarrheagenic *E. coli* pathotypes is very important since they are involved in cancer or cellular senescence. It was concluded that at attempts at economy are better conducted via finding appropriate criteria for requesting a stool culture rather than at limitation of the microbiological evaluation of specimens that are subjected.**

Key words: Serogrouping; Multiplex PCR; Detection; Escherichia *coli*

Escherichia coli are commensal bacteria of the human gut microbiota, but some pathogenic strains have acquired the ability to induce chronic inflammation and/or produce toxins, such as cyclomodulin, which could participate in the carcinogenesis process. It was explained that the *E. coli* population associate with mucosa of patients with colon cancer in relation to clinicopathologic characteristics [1].

The widespread species *Escherichia coli* are categorized into six pathotypes, which are associated with diarrhea and collectively are referred to as diarrheagenic *E. coli*: enterotoxigenic *E. coli* (ETEC), which produces one or more enterotoxins that are heat labile LT (*lt*) or heat stable ST (*st*); enteropathogenic *E. coli* (EPEC), which has a pathogenicity island that encodes a series of proteins involved in the attaching and effacement lesions of the intestinal microvilli of the host cell; and the presence of the large EPEC adherence factor (EAF) plasmid (*eae*), on which also the cluster of genes encoding bundle-forming pilli (*bfp*) is present. Based on these, EPEC strains are classified as typical when they possess the EAF plasmid, whereas atypical EPEC strains don't possess the EAF plasmid; enterohemorrhagic (EHEC) or Shiga toxin producing *E. coli* (STEC) is characterized by the production of two strong cytotoxins denominated Shiga-like toxins 1 and 2

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(*stx1* and *stx2*). Like EPEC, EHEC elicits an A/E lesion of the intestinal mucosa, a phenotype that requires a functional *eaeA* chromosomal gene [2, 3].

The three other categories seem to be less prevalent. Enteroinvasive *E. coli* (EIEC) has biochemical, physiological, and genetic properties similar to those of Shigella, invading the epithelial cells of the colon, where it proliferates and causes necrosis of the tissue. Invasion plasmid antigen H (*ipaH*) found in EIEC and *Shigella*; [3] enteroaggragative *E. coli* (EAEC) strains are characterized by an aggregative adherence (AA) pattern on cultured epithelial cell and produce fimbrial colonization factors called aggregative adherence factors (AAF) [4]. The most recently characterized category corresponds to diffusely adherent *E. coli* (DAEC), strains that are capable of adhering to HEP-2 cells in a nonlocalized pattern. The "gold standard" method for detection of DAEC strains is based on the diffusely adherent pattern in the HEp-2 adherence assay or by detection of the gene *daaE* that is necessary for the expression of the F1845 ûmbriae [2].

These *E. coli* pathotypes are commonly isolated from patients with gastroenteritis in the developing world. However, they are not routinely sought as stool pathogens in clinical laboratories. Some of these pathogens respond to antimicrobial agents, while for others (e.g., Shiga toxin-producing *E. coli* [STEC]), antibiotics should be avoided. Since the time frame in which treatment choices must be made is short, there is a need for a rapid, sensitive, and inexpensive detection technique [5].

Infections caused by diarrheagenic *E. coli* (DEC) strains are important; therefore, it is important to detect them at the onset and pursue necessary preventive measures. For many years, laboratory detection of food–borne pathogens has relied on direct isolation, which is still the preferred method, as it represents the gold standard as compared to newly developed rapid detection methods. However, several methods available for rapid detection are discussed in order to shorten the detection time and begin therapeutic measures[6, 7].

Among all diagnostic techniques such as culture, serology and molecular methods, the latter has high sensitivity, specificity and safety. Nonetheless, culture and serology methods remain popular because of their simplicity. Serotyping and biochemistry have been widely applied in the diagnosis of gastrointestinal pathogens, but they cannot be used for definite detection of DEC groups. Therefore, identiûcation of the characteristic virulence genes is a better choice for DEC diagnostics [6, 8].

Little is known about the prevalence of *E. coli* pathotypes in Iran. It has been estimated that diarrheagenic *E. coli* are among the most prevalent causative agents in acute diarrhea [9, 10]. However, there are no well-documented mortality figures for *E. coli*-induced disease, because the microbiologic diagnosis cannot be done easily in many settings. *E. coli*-related deaths at present would be counted as diarrheal and colon cancer deaths in many countries [1]. So, knowledge of the status of the *E. coli* pathotypes in Iran is important for planning appropriate public health programs for controlling the disease. Therefore, the aim of this study was to evaluate the usefulness of serology and PCR methods in detecting the *E. coli* strains isolated in Iran.

MATERIALS AND METHODS

Clinical specimens

One hundred and fourty seven *E. coli* isolates were obtained from the cases of diarrhea in the hospitals of Tabriz (Iran) within 2013-2014. These isolates were identified by conventional methods and stored at -70°C in TSB (Merck, Germany) restraining 20% glycerol until required [12].

Bacterial strains

Diarrheagenic *E. coli* reference strains 933J (*stx1, stx2, eae*), 2348/69 (*eae, bfp*), H10407 (*st, lt*), O42 (*ipaH*), and F-1845 (*daaE*) were used as positive controls. One hundred and forty seven *E. coli* strains were analyzed, including strains representing all of the currently recognized six classes of diarrheagenic *E. coli* as well as the commensal organisms. The prototypical strains currently used in the laboratories worldwide include enterotoxigenic *E. coli* (ETEC) H10407; enteropathogenic *E. coli* (EPEC) 2348/69; enteroaggregative *E. coli* (EAEC) O42; *E. coli* (EIEC) EI-34 and *E. coli* (DAEC) F-1845 (*daaE*). Reference strains were provided by the Center for Vaccine Development (CVD), University of Maryland; MP, Microbiology Program, University of Chile.

Extraction of DNA

A sweep of about five *E. coli* like colonies was cultured in Tripticase Soy Broth (TSB) for 18h. The DNA was extracted using Promega genomic DNA extraction kit according to the manufacture's protocol (A11125, USA).

PCR primers design

PCR primers were designed based on the virulence gene sequences resumed from the National Center for Biotechnology Information (NCBI) database [2]. The obtained amplicons ranged from 100 to 900 bp in length. All primers were obtained from GeNetBio Inc., Korea (Table 1).

Multiplex PCR assays

The multiplex PCR assays were carried out using a 2X PCR Master Mix, which is an optimized premixed with 2X concentrated solutions of Taq DNA polymerase, reaction buffer, $MgCl_2$ and dNTPs. The 2X PCR Master Mix contains all components for PCR, except DNA template and primers. The DNA templates were subjected to multiplex PCR with specific primers (Table 2). Three multiplex PCRs were standardized for identification of the DECs.

Multiplex PCR assay 1

The reaction mixture of optimized protocol was carried out with a 25 ìl mixture of 2X PCR Master Mix containing: 5 ìl of the DNA template and 1 ìM of each of the primers (GeNetBio Inc., Korea), i.e. *lt* and *st* for ETEC isolates, and *ipaH* for EIEC isolates. The cycling conditions in BioRad T100™ thermal cycler were as follows: 95°C for 5 min for one cycle followed by 35 cycles of 95°C for 45 sec, 49°C for 45 sec, and 72°C for 1 min and 72°C for 10 min.

Multiplex PCR assay 2

The protocol used was as explained above for multiplex PCR 1 except that the primers were *eae* and *bfp* for EPEC isolates, and *eae, stx1 and stx2* for EHEC isolates. The thermocycling conditions were as follows: 95°C for 5 min for one cycle followed by 38 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 1min and 72°C for 10 min.

Multiplex PCR assay 3

The multiplex PCR assay 3 was performed with the *daaE* and *aggR* (transcriptional activator for the AAFs) primers for DAEC and EAEC. The ampliûcation conditions were as follows: an initial denaturation step at 95°C for 5 min was followed by 39 cycles of 95°C for 45 sec, annealing at 55°C for 45 sec and elongation at 72°C for 1 min. A ûnal elongation step was executed at 72°C for 10 min. PCR products were evaluated on a 1.5 % (w/v) agarose gel (Ultrapure Agarose). The DNA bands were visualized under UV light after staining the gel with ethidium bromide (Fermentas Inc., Maryland, USA) [3, 13].

Serotyping

Serotyping is based on the use of specific antisera and the detection of somatic O and K antigens expressed by *E. coli* pathotypes (Table 1). O-K polyclonal antisera (Sifin Kit) were used for slide agglutination with live cultures, and the reaction was observed after 5-10 sec [14].

Antibiotic susceptibility tests

Susceptibility testing was conducted by Kirby-Bauer disk diûusion according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Susceptibility of *E. coli* isolates was tested to the commonly used antibiotics such as Cefoxitin, Ceftazidime, Cefotaxime, Cefepime, Gentamicin, Kanamycin, Co-trimoxazole, Ampicillin, Imipenem, and Ciproûoxacin (Table 3). *E. coli* ATCC 25922 was used as quality control [11, 15].

RESULTS

All *E. coli* strains were serogrouped by a latex agglutination test using a commercial antiserum. As demonstrated in Table 1, of 147 *E. coli* isolates subjected to latex agglutination test, 53 isolates were positive, and according to the kit regents, three different groups were identified; 13 (8.84%) strains showed positive reaction with antiserum 1, and in 10 (6.80%) strains, agglutination with antiserum 2 was observed. Also, in 30 (20.4%) strains, positive response was identified in antiserum 3. It was detected that 6 ETEC, 4 EPEC, 5 EHEC, 5 EAEC and 1 EIEC were not typeable with O-K polyclonal antisera. As evident in Tables 1 and 4, the most commonly detected *E. coli* seroroups were O25, O78, O103, O118, O124, O145, O157 and O164, while 23 strains were untypeable by the available antisera. As a result, the highest percentage of ETEC, EPEC and EHEC isolates showed positive response with antiserum 3. One of the *E. coli* isolates showed positive reaction with all of the kit reagents, but no virulence factor genes were isolated in the PCR assays. Also seven

isolates were positive in the latex agglutination test; however, virulence genes were not detected with our primers.

To demonstrate the utility of multiplex PCR assays, 147 *E. coli* strains isolated from diarrheic patients were subjected to the multiplex PCR, and the results were compared with those obtained by single PCRs (Table 4). The PCR assays

Table 1. Diagnostic polyvalent antisera in Sifin Kit

Serogroup	Antiserum
Anti-coli 1	O26:K60; O44:K74; O114;K90; O125;K70; O142:K86; O158;K-
Anti-coli 2	O55:K59; O86:K61; O91:K-; O111:K58; O119:K69; O126:K71; O127:K63; O128:K67
Anti-coli 3	O25:K11; O78:K80; O103:K-; O118:K-; O124:K72; O145:K-; O157:K-; O164:K-

Table 2. Primers used in the multiplex PCR for amplification of diarrhagenic *E. coli* genes

Target organism	Target gene	Genom location	Primers (5'-3')	Product size (bp)
			ATT TTT ATT TCT GTA TTA TCT T	
ETEC	*st*	Plasmid	C CAC CCG GTA CAT GCA GGA TT	190
	lt	Plasmid	GGC GAC AGA TTA TAC CGT GC CGG	450
			TCTCTA TAT TCC CTG TT	
EPEC	*eae*	Chromosome	AGG CTT CGT CAC AGT TG	570
			CCA TCG TCA CCA GAG GA	
	bfp	Chromosome	AATGGTGCTTGCGCTTGCTGC	326
			GCCGCTTTATCCAACCTGGTA	
EHEC	stx_1	Phage	AGA GCG ATG TTA CGG TTT G	388
			AGA GCG ATG TTA CGG TTT G	
	stx_2	Phage	TGG GTT TTT CTT CGG TAT C	807
			GAC ATT CTG GTT GAC TCT CTT	
EIEC	*ipaH*	Plasmid	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	900
			GCC GGT CAG CCA CCC TCT GAG AGT AC	
EAEC	*aggR*	Plasmid	cta att gta caa tcg atg ta	457
			ga gtc cat ctc ttt gat aag	
DAEC	*daaE*	Chromosome or plasmid	GAACGTTGGTTAATGTGGGGTAA	
			TATTCACCGGTCGGTTATCAGT	

Table 3. Antibiogram of *E. coli* pathotypes isolated from the cases of diarrhea

		Number of isolates (n=63);Percentage in parenthesis		
Antimicrobial agent	Disk content (µg)	Resistant	Intermediate	Susceptible
Ampicillin	10	68(100)	0	0
Penicillin G	10	68(100)	0	0
Ciprofloxacin	5	8(11.76)	0	60(88.23)
Ceftazidime	30	4(5.88)	16 (23.52)	48(66.66)
Gentamycin	10	2(2.94)	0	66(97.05)
Kanamycine	30	4(5.88)	56(82.35)	8(11.76)
Nitrofurantoin	30	0	0	68(100)
Nalidicsic acid	30	18(26.47)	0	50(73.52)
Chloramphenicol	30	15(22.05)	9(13.23)	44(64.70)
Trimethoprim-Sulphamethoxazole	1.25/23.75	56(82.35)	0	12(19.04)
Imipenem	10	0	0	68(100)

detected 26 ETEC, 20 EAEC, 11 EHEC, 9 EPEC, and two EIEC isolates. No DAEC strains were isolated from any of the samples examined. There was agreement between multiplex PCR and single PCRs assays for all strains. As demonstrated in Table 3, the multiplex PCR showed positive results for 68 diarrheagenic *E. coli* strains. The specificity of multiplex PCR was tested with the reference strains. Non-specific bands were not visualized (Figures 1, 2, 3). Two of the *E. coli* strains were positive for the ETEC *lt* marker plus additional virulence factors not typical of ETEC. One of the strains was positive for *aggR*, a known marker of EAEC. The second strain was positive for *ipaH*, which is EIEC marker.

The antibiotic susceptibility profile of the detected *E. coli* pathotypes is presented in Table 3. About 100% of the pathotypes were sensitive to Imipenem and Nitroforantoin, while susceptibility to other antibiotics was in the following order: Gentamycin 97.05%, Ciprofoxacin 88.23%, Nalidicsic acid 73.52%, Ceftazidime 66.66%, Chloramphenicol 64.70%. All of the pathotypes were 100% resistant to Ampicillin and Penicillin G, while 82.35% of the pathoypes were resistant to Trimethoprim-Sulphamethoxazol.

DISSCUSSION

Phenotypic assays such as serogrouping with traditional antiserum are the routine methods widely used in clinical laboratories [16, 17]. Serotypic markers correlate, sometimes, extremely with speciûc categories of diarrheagenic *E. coli*; however, they are rarely enough in themselves to identify strains reliably as diarrheagenic. Thus, the

Table 4. Frequencies of serogroups associated with *E. coli* pathotypes

			No. of agglutination with kit reagents (Percentage)			
			Anti 1	Anti 2	Anti 3	NT
Pathotypes	Virulence genes	No. of pathotypes in PCRs				
ETEC	*lt*	26(17.68)	7(26.92)	4(15.38)	9(34.61)	6(23.07)
	st					
	lt,st					
EHEC	*eae,stx-1*		0	1(9.09)	4(36.36)	6(54.54)
	e*ae,stx-2*					
	eae,stx-1,stx-2	11(7.48)				
	stx-1					
	stx-2					
EPEC	*eae*	9(6.12)	0	1(11.1)	4(44.4)	4(44.4)
EIEC	*ipaH*	2(1.36)	0	0	1(50)	1(50)
EAEC	*aggR*	20(13.60)	6(30)	1(5)	8(40)	5(25)
DAEC	*daaE*	0				

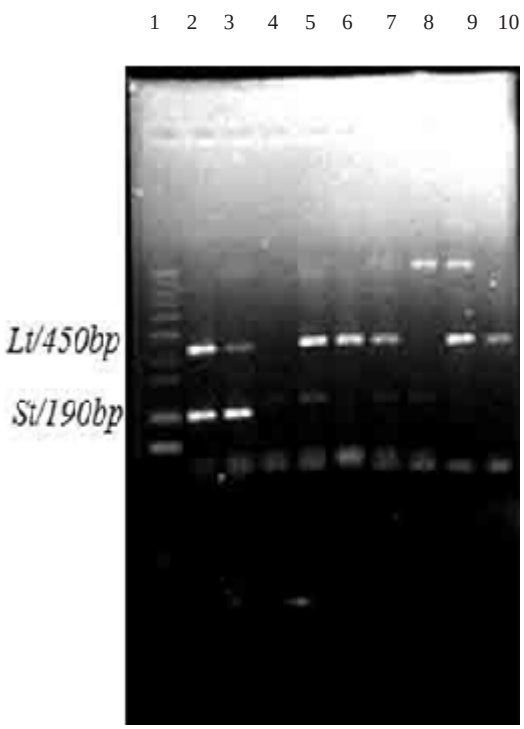

Fig. 1. Multiplex PCR of ETEC and EIEC reference strains and clinical samples. Lane 1: DNA molecular size marker (100bp ladder), Lane 2: ETEC (*lt* amplicon size 450 and *st* amplicon size 190 bp), Lanes 3, 5, 7: Clinical isolates of ETEC (*lt, st*), Lane 4: Clinical isolates of ETEC (*st*), Lane 6: Clinical isolates of ETEC (*lt*), Lane 8: EIEC (*ipaH* amplicon size 900 bp), Lane 9: Clinical isolates (*lt, ipaH*).

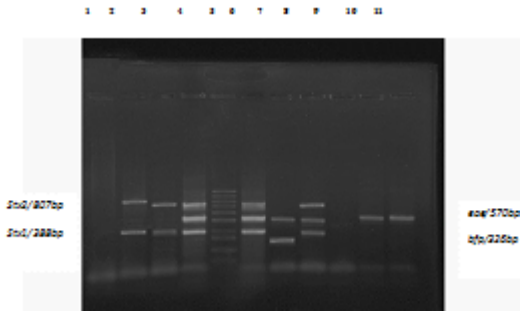

Fig. 2. Multiplex PCR of EHEC and EPEC reference strains and clinical samples. Lanes 1, 9: Negative control, Lanes 2, 3: Clinical isolates of EHEC (*stx1*, *stx2*), Lanes 4, 6: EHEC (*stx1*, *eae* , *stx2*), Lane 5: DNA molecular size marker (100bp ladder), Lane 7: EPEC (*eae*, *bfp* amplicon size 326 bp), Lane 8: Clinical isolates of EHEC (*stx1* amplicon size 388, *eae* amplicon size 570 and *stx2* amplicon size 807bp), Lane10: aEPEC (*eae*), Lane 11: Clinical isolates of aEPEC (*eae*)

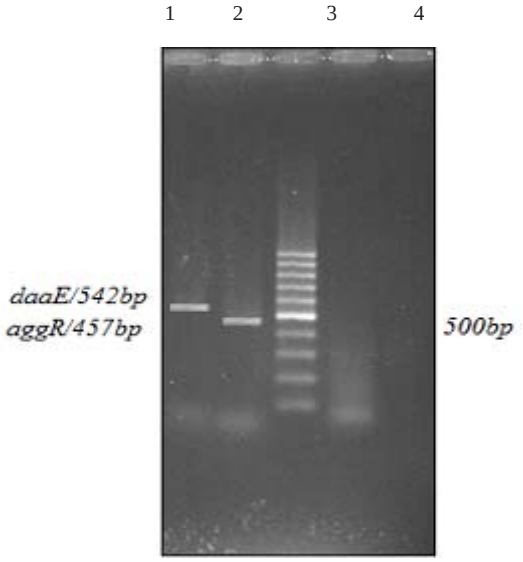

Fig. 3. Multiplex PCR of EAEC and DAEC reference strains. Lane 1: EAEC (*aggR* amplicon size 457 bp), Lane 2: DAEC (*daaE* amplicon size 542 bp), Lane 3: DNA molecular size marker (100bp ladder), Lane 4: Negative control.

finding of diarrheagenic *E. coli* has focused more and more on the identiûcation of certain characteristics based on the presence of different chromosomal and/or plasmid encoded virulence genes that are not present in commensal *E. coli* [9, 17].

Vaishnavi *et al.*, [19] reported that of 100 *E. coli* isolates only 25 were typeable and belonged to 14 different O-serogroups. They were 5 O153, 4 O102, 3 O25, 2 each of O130 and O169, and 1 each of O1, O8, O15, O37, O86, O101, O127, O143, and O160. In a study in Iran, the result of O-serogrouping by EPEC antisera showed that 17 (5.3%) isolates of the 321 *E. coli* isolates were typeable with the used antisera, and it was deduced that only 65% of the serogrouped EPEC strains carried virulence genes [10]. In a study in Kenya, it was shown that 76% of *eaeA* positive *E. coli* strains belonged to traditional EPEC serogroups [19].

In the present study, the positive rates of pathogenic genes in the classical serogroup based *E. coli* strains were examined. According to our results and other studies, there was a relation between serogroup and pathogenic genes, but discovery of the virulence genes by PCR was more successful in detection of DEC. In this study,

36.05% of the *E. coli* isolates were serogrouped by a latex agglutination test, while 46.25% of the isolates were detected to harbor virulence genes using PCR assays. It was also deduced that the association of stereotyping with PCR assay was statistically significant.

PCR analysis for identiûcation of DEC groups has been designed to detect one or a few genes per reaction. However, multiplex PCRs are now being planned to detect multiple genes in the same reaction, thereby further reducing the cost and time required for the experimental procedure [7, 20]. In the present study, the direct challenge of standardizing three multiplex PCR assays is the probability for primer dimmers and nonspecific products. So, it is important to design primers with close annealing temperatures, and to use reference strains to find reaction specificity. Our results confirm that this technique can be administered for diagnosis of patients with the diarrhea, and the multiplex PCR assays showed high specificity for future epidemiological studies of DEC. This is an important observation from a public health point of view because clinical laboratories in Iran do not use molecular techniques for detection of DEC.

Many PCR assays have been developed to identify the virulence genes of DEC. Toma *et al.*, [8] showed that it is possible to detect 11 of the major virulence genes of *E. coli* with four multiplex PCRs. For each of the target genes, various pairs of primers were selected from the studies. Also in a study by Hegde *et al.*, 20 two multiplex PCRs were standardized for identification of all DECs. They found that EAEC was the most common DEC usual in children with diarrhea in India [21]. EAEC has also been reported as the leading DEC in children in studies carried out in Brazil [22]. EAEC has been reported to cause traveler's diarrhea and persistent diarrhea in children in the developing countries with 19% reported in Goa and 33% in Guadalajara [4, 15]. These results are similar to our study, in which 13.7% of the *E. coli* isolates identified as EAEC. However, according to our study, with the exception of DAEC strain, which was not detected in 147 *E. coli* isolates, we were able to differentiate five categories of diarrheagenic *E. coli*, including the less common EIEC and variety of different serogroups of ETEC, EHEC and EPEC. The most frequent category of diarrheagenic *E. coli* detected was ETEC, which accounted for 17.8% of the isolates. This was followed by EAEC (13.7%); EHEC (7.5%), EPEC (6.2%) and EIEC (1.4%). These data corroborate the previously reported prevalence data, in which ETEC strains were amongst the commonest causes of intestinal disease in different parts of the world [2, 23]. Also there were two strains, which possessed *lt* plus *ipaH* and *aggR* genes, indicating that these two strains are a combination of ETEC-EAEC and ETEC-EIEC pathotypes. Some researchers believe that the virulence plasmid of EAEC and EIEC strains may have horizontally transferred to ETEC and EPEC recipient strains or vice versa [3, 24].

Tsukamoto and Kawai [25] showed that the percentage of EPEC in the *E. coli* strains carrying *eaeA* was about 55% and according to Albert *et al.*, [26] 57% of EAF-positive and/or *eaeA* positive EPEC strains belonged to a traditional serogroup. In the present study, the prevalence of tEPEC and aEPEC was 6.2%. The gene encoding *eaeA* was detected in 2.7% of the strains, and the *bfp* gene on the EAF plasmid was found only in 5 strains. According to our results, the rate of EPEC was similar to the study by Karam *et al.*, in Iran, [10] but it was less than other reports (8.4%) from Iran [27]. Furthermore, only 5 of the EPEC strains were typeable with used antisera. One of the typical EPECs and 3 atypical EPEC were identified with antiserum 3, and only 1 atypical EPEC was positive with antiserum 2. In a study by Sunabe and Honma, [28] it was reported that there was no considerable difference between *eae* positive rates in the serogroup-based EPECs and EIECs. In the present study, the *ipaH* was detected in 2 of the *E. coli* isolates, and only one of the EIEC strains was serogrouped by aniserum 3. According to other studies, many of EHEC strains also have the *eae* gene; so presence of *stx* genes can distinguish EHEC strains from EPEC strains [19, 29]. In our study, 11 EHEC strains were detected with the used primers. Seven of them had the stx_2 gene, the frequent toxin phenotype pattern described in the Northern hemisphere. However, 4 strains harbored stx_1 gene. It is to be mentioned that the prevalence and other geographical features of these pathogens as leading cause of diarrhea vary from country to country. The results of this study show that the validity of diagnosis with seroagglutination test needs to be addressed because O antigenic markers, sometimes, do not associate with

pathogenicity of an isolate.

Also, the *E. coli* found in this study expressed high levels of resistance to antimicrobials that are usually used in clinical medicine. This approves the fact that in most of the developing countries, diarrheal diseases are treated with insufficient regimen antibiotics and often without first identifying the pathogen [4, 27]. In Iran, the cost of materials needed to detect *E. coli* is about $5.00 per stool sample, and in this period of intensive care, if even one surgical procedure is obviated by the accurate diagnosis of *E. coli* infection or if even one severe secondary case of *E. coli* infection and colon cancer can be prevented, the cost of screening hundreds of stools for *E. coli* pathotypes can be justified [4].

CONCLUSION

In conclusion, differentiation between the diarrheagenic *E. coli* pathotypes is very important since they are involved in cancer or cellular senescence. So, this study helped to realize that DEC does contribute to the burden of diarrhea in people in the developing world [4, 15]. It was concluded that at attempts at economy are better conducted via finding appropriate criteria for requesting a stool culture rather than at limitation of the microbiological evaluation of specimens that are subjected.

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