



Enter aggregative *Escherichia coli* (EAEC): An Emerging Enteric Pathogen in South of Iran

Pejman Abbasi^{1*}, Mohammad Kargar^{2*}, Abbas Doosti³, Jalal Mardaneh¹, Sadegh Ghorbani-Dalini⁴, Mohammad Ali Dehyadegari¹

¹Professor Alborzi Clinical Microbiology Research Center, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, IR Iran.

²Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, IR Iran.

³Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, IR Iran.

⁴Jahrom Branch, Young Researcher's and Elit Club, Islamic Azad University, Jahrom, IR Iran.

ARTICLE INFO

Article type:

Original Article

Article history:

Received: 20 Aug 2014

Revised: 8 Sep 2014

Accepted: 21 Sep 2014

Keywords:

Diarrhea,

Enter aggregative

Escherichia coli

Real-Time PCR

ABSTRACT

Background: Enter aggregative *E. coli* (EAEC) is increasingly recognized as a cause of often persistent diarrhea in children and adults in both developing and developed countries. The aim of the present study was to investigate the presence and the frequency of EAEC as etiologic agent of diarrhea in Shiraz.

Methods: A total of 715 stool samples were collected from patients with diarrhea in Shiraz. Diarrheagenic *E. coli* were isolated by biochemical tests and culture from 715 stool samples collected from different hospitals. Diarrheagenic *E. coli* strains isolated from diarrheal stool samples were examined for the detection of the *aggR* gene by Real time PCR and PCR method.

Results: In this study, a total of 101 (14.12%) diarrheagenic *E. coli* were isolated from 715 stool samples collected from different hospitals. The infected patients were 58 (57%) males and 43 (43%) females. Out of these 101 diarrheagenic *E. coli* identified, 5 were confirmed as EAEC in patient. The EAEC strains were isolated from 3 of the 43 females (43%) and 2 of the 58 males (57%) with the mean age of 11.4±1.2 age. In this study, 5 EAEC strains were isolated from one patient with bloody diarrhea and 4 patients with watery diarrhea. The high prevalence of EAEC isolates was also found in watery diarrhea.

Conclusion: We therefore, recommend the routine isolation and identification of EAEC strains from patient with diarrhea in all the clinical laboratories and other pathotype diarrheagenic *E. coli* in Iran.

- **Please cite this paper as:** Abbasi P, Kargar M, Doosti A, Mardaneh J, Ghorbani-Dalini S, Dehyadegari MA. Enter aggregative *Escherichia coli* (EAEC): An Emerging Enteric Pathogen in South of Iran. *J Med Bacteriol.* 2014; 3(3, 4): pp.40-46.

Introduction

Diarrhea is a leading cause of morbidity and mortality in all age groups and areas of the world (1). In the past decades several new enteric pathogens, including bacterial, viral, and parasitic agents have been described. However, since most of the burdens of diarrheal diseases are incurred among infants and children in developing countries, the majority of diarrhea etiology studies are performed on children in such regions (2). *E. coli*, a member of the bacterial family of Enterobacteriaceae, is the most prevalent commensal inhabitant of the gastrointestinal tracts of humans and warm-blooded animals, as well as one of the most important pathogens (3, 4). Diarrheagenic *Escherichia coli* (DEC) strains are an important cause of diarrhea among children in developing countries and are now being recognized as emerging enteropathogens in the developed world. Six *E. coli* pathotypes are currently known: enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and Shiga toxin-producing *E. coli* (STEC), and enterohemorrhagic *E. coli* (EHEC). EPEC strains are divided into typical (EPEC) and atypical EPEC (aEPEC) strains based on the presence or absence of the EAF plasmid, respectively (5). Enteroaggregative *Escherichia coli* (EAEC) is a category of diarrheagenic *E. coli* defined by a characteristic “stacked brick”, “honeycomb” or “aggregative” adherence pattern to epithelial cells (6). EAEC are increasingly recognized as a cause of often persistent diarrhoea in children and adults in both developing and developed countries, and have been identified as the cause of several outbreaks worldwide (7, 8). EAEC is defined by its characteristic “stacked brick” aggregative pattern of adherence to HEP-2 cells. In addition, several possible EAEC virulence factors have been reported. However, the pathogenic mechanisms of EAEC infection are not fully understood.

Moreover, there appears to be significant heterogeneity of virulence among EAEC isolates (9). The pathogenesis of EAEC is really complex as strains are relatively heterogeneous. The best-studied virulence factor is *aggR*, the master regulator of EAEC virulence, which controls expression of adherence factors, a dispersin protein, and a large cluster of genes encoded on the EAEC chromosome (10). We chose to amplify *aggR* gene because it may well be the most reliable indicator of a truly pathogenic EAEC strain. The increasing number of such reports and the rising proportion of diarrheal cases in which EAEC are implicated suggest that EAEC are important emerging agents of paediatric diarrhea (11), but in Shiraz, the status of EAEC prevalence and contribution to disease is uncertain. The aim of the present study was to investigate the presence and the frequency of EAEC as etiologic agent of diarrhea in Shiraz.

Materials and Methods

Clinical specimens and culture process

A total of 715 stool samples were collected from patients with diarrhea in Namazi hospital and Shahid Dastgheyb hospital Shiraz, in 2012. Fecal sample, from patients were transported to the laboratory in PBS transport mediums on ice packs. A loop full of diarrheal sample was streaked on MacConkey agar and incubated for 24 h at 37 °C, pink colonies then sub cultured on Eosin Methylene Blue (EMB) on which the colonies exhibit green metallic sheen color, for a further conformation set of biochemical tests were used.

DNA extraction and Primer selection

Subsequently, a sweep of three colonies were inoculated in Luria-Bartani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37°C with shaking.

All isolated *E. coli* strains were grown on Luria-Bertani agar (Sigma, St. Louis, MO) over night at 37°C. *E. coli* genomic DNA was extracted using DNA extraction kit (QIAGEN Ltd., Crawley, UK) according to manufacturer's instructions. The forward (5'-CGAAAAAGAG ATTATAAAAATT AAC-3') and reverse (5'-GCTTCCTTCTTTTGT GTAT-3') primers were selected for detection *aggR* virulence gene in isolated diarrhegenic *Escherichia coli* (DEC) strains (12). The expected length of the amplified product of the target sequence with these primers was 100 bp.

PCR assays

Each PCR assay was performed with a final reaction volume of 25 µl containing 2 µl of the template DNA, 200 mM deoxynucleoside triphosphates, 4 mM MgCl₂, 1.5 unit U Taq DNA polymerase (sinagen, Iran), 0.2 mM of each primer. Cycling parameter was used as follow: 95 for 5 min to initially denature the DNA, then 35 cycle consist of 1 min at 94 °C, 1 min at 58 °C to, 1 min at 72 °C, and finally single prolonged extension at 72 °C for 5 min. A negative control lacking the DNA template was included in each experiment to exclude the possibility of the reagent contamination. The *E. coli* strain used as control in the PCR test included enteroaggregative *E. coli* O42. The amplified product was visualized by gel electrophoresis in 1.5% agarose gel containing ethidium bromide for 45 min at 100 V and then visualized under UV light (Figure. 1).

Real time PCR assays

Real-time PCR assay for detection of EAEC strains was conducted in a final volume of 25µl as same as PCR plus 1µl of SYBR Green I (In vitrogen, USA). Reactions were performed on Rotor-Gene 6000 (Corbett Research, Australia) by cycling condition of 95 °C for 5 min followed by 45 cycles of 95°C for 30 s and 58°C for 40 s. Finally, melt curve analysis was performed from 70-99°C with ramping rate of 2.5°C/s and analysis of fluorescence at each 2 °C for 5s. All reactions

were repeated in triplicates and positive and negative control samples were used in each run. All data were analyzed by rotor-gene 6000 software version 1.7.

Data analysis

Ax² test or Fisher's exact test was used to determine the statistical significance of the data. A *P* value of <0.05 was considered significant.

Results

In this study, a total of 101 (14.12%) diarrhegenic *E. coli* were isolated by biochemical tests and culture from 715 stool samples collected from different hospitals. There were 58 (57%) males and 43 (43%) females with the mean age of 13.523±1.662 age (ranged from 2 months to 63 years). Diarrhegenic *E. coli* were isolated much more frequently in the summer months than other season. The patients were categorized into two groups according to their age: (0-5 year) and Upper 6 year. The EAEC strains were isolated from 3 of the 43 females (43%) and 2 of the 58 males (57%) with the mean age of 11.4±1.2 age. The organism is isolated from children more frequently than from persons in other age groups and from female more frequently than males (Table 1). A total of 5 EAEC strains were observed in dry season samples (Table 1). In this study, 5 EAEC were isolated from one patient with bloody diarrhea and 4 patients with watery diarrhea. The high prevalence of EAEC isolates was also found in watery diarrhea (Table 1).

Table 1. Distribution of 5 EAEC strains in accordance to seasons, age, sex and clinical symptoms.

Clinical and other characterization		EAEC. No
Season	Spring	Not seen
	Summer	5
	Fall	Not seen
	Winter	Not seen
Age	(0-5 year)	3
	(6-61 year)	2
Sex ratio (M/F)	Male	2
	Female	3
Clinical symptoms	Diarrhea Watery	4
	Diarrhea Bloody	1
	Fever	2
	Vomiting	3

In this study, a total of 101 *E. coli* strains isolated from diarrheal stool samples were examined for the detection of the *aggR* gene by traditional PCR method (Fig. 1) and Real time PCR assay (Fig. 2). Out of these 101 diarrheagenic *E. coli* identified, 5 were confirmed as EAEC in patient. The results obtained from standard PCR and Real-time PCR was same.

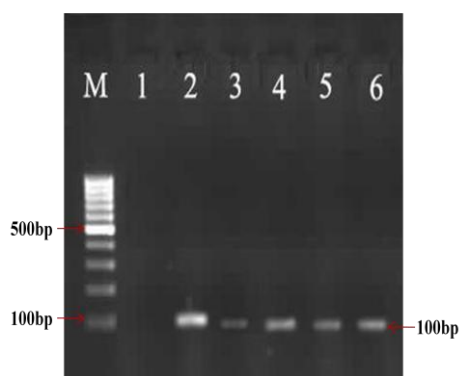


Figure 1. Agarose gel of amplicons from the PCR. The molecular weight ladder is shown in lane M; strain identification and amplicons are shown in lanes 2 to 5; nonpathogenic *E. coli* is shown in lane 1; *E.coli* O42 is shown in lane 6.

DISCUSSION

Enteroaggregative *Escherichia coli* (EAEC) is a subgroup of diarrheagenic *E. coli* (DEC) that during the past decade has received increasing attention as a cause of watery diarrhea, which is often persistent (11). In the present study, EAEC were detected in 5 (4.95%) patients with diarrhea. We report the first study performed in Shiraz to identify EAEC in patient with diarrhea. Our finding is approximately similar to that reported by Jafari *et al*, in which they found that the rate of EAEC was 5.4% (13). Several other outbreaks both in children and in adults have been described in the UK, and France (10). Thus in this study, the patients were categorized into two groups according to their age: 0-5 years and Upper 6 years. In the present study, EAEC is isolated from children 0-5 years more frequently than from patients in the Upper 6 years. Our finding is approximately similar to that reported by Pourakbari *et al*, in Tehran (14) and Alikhani, *et al*, in Hamadan (2).

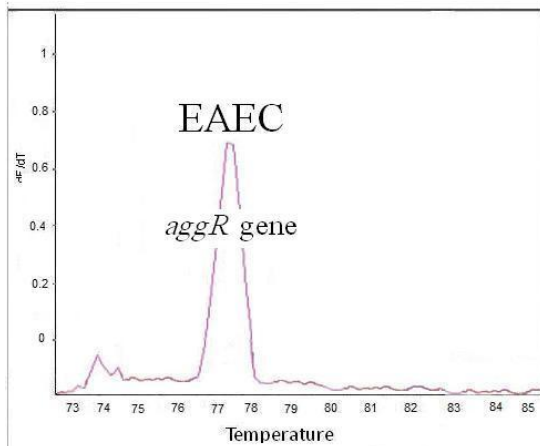


Figure 2. Detection of EAEC pathotype by Multiplex Real-time PCR on *aggR* gene.

In Brazil, studies showed EAEC as an important agent of acute diarrhea in children under 5 years old (16). Other study from Brazil identified EAEC infection as the most common cause of diarrhea in small children, and was found to be more frequently associated with diarrhea in children (10). Our results agree with these studies and show that, EAEC strains are one of the enteric pathogens in children. The features of this pathogen as causative agents of diarrhea vary from place to place depending on local meteorology, geography and socioeconomic variables. This variation is also seen between and within countries in the same geographical area (17). Although not all EAEC infections result in symptomatic illness, the most commonly reported symptoms are watery diarrhea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever (10). In this study the high prevalence of EAEC isolates was also found in watery diarrhea and other symptoms were also observed. In this study, 5 EAEC were isolated from one patient with bloody diarrhea and 4 patients with watery diarrhea. Our results showed that high prevalence of EAEC isolates was also found in watery diarrhea.

Also, 5 EAEC was isolated from 2 patients with fever and from 3 patients with vomiting. Our finding is approximately similar to that reported by Jafari *et al* in Tehran (13). In present study, sampling was performed in dry season (summer). Jafari *et al*, have reported prevalence of EAEC in summer, but in other study by Pourakbari *et al* (14), no difference in the rate of detection of diarrheagenic *E. coli* proportion was found between the dry (summer) and rainy season (autumn) samples (18). There is a paucity of data concerning this pathogen in Iran due to the unavailability of a simple, easy to perform and cost-effective test for its detection. Therefore of the few studies that have dealt with diarrheagenic *E. coli*, almost all have used PCR method for detection of EAEC, either targeting the *aggR* sequences (19, 20, 22). The Real-time PCR assay is rapid, sensitive, and specific method for the detection of all diarrheagenic *E. coli* pathotypes such as EAEC in routine diagnostic laboratories (23). Real-time PCR measures the amount of the product during the exponential phase whereas traditional PCR measures product during the plateau phase. It is more effective to measure during the exponential phase because measurements taken during the plateau phase do not always clearly indicate the quantity of starting material. Traditional PCR is time-consuming and laborious processes because requires post-PCR analysis, possibly agarose gel electrophoresis; and it identifies the product either by size or sequence (12). Another advantage of real-time PCR over traditional PCR is that the entire process from amplification to analysis is performed in the same tube. This differs from traditional PCR where the PCR product is moved and manipulated into other formats. As a result, there is a decreased possibility of contaminating the product with real-time PCR methods. We therefore, recommend the routine isolation and identification of EAEC strains from patient with diarrhea in all the clinical laboratories and other pathotype diarrheagenic *E. coli* in Iran.

Acknowledgments

The authors thank Islamic Azad University, Jahrom Branch (Jahrom, IR Iran) for the financial support.

Conflict of interest

None declared conflicts of interest.

References

- Fischer Walker CL, Sack D, Black RE. Etiology of diarrhea in older children, adolescents and adults: a systematic review. *PLoS Negl Trop Dis* 2010; **4**: e768.
- Alikhani MY, Hashemi SH, Aslani MM, Farajnia S. Prevalence and antibiotic resistance patterns of diarrheagenic *Escherichia coli* isolated from adolescents and adults in Hamedan, Western Iran. *Iran J Microbiol* 2013; **5**: 42-7.
- Allocati N, Masulli M, Alexeyev MF, Di Ilio C. *Escherichia coli* in Europe: An Overview. *Int J Environ Res Public Health* 2013; **10**: 6235-54.
- Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 1998; **11**: 142-201.
- Souza TB, Lozer DM, Kitagawa SM, Spano LC, Silva NP, Scaletsky IC. Real-time multiplex PCR assay and melting curve analysis for identifying diarrheagenic *Escherichia coli*. *J Clin Microbiol* 2013; **51**: 1031-33.
- Okeke IN, Wallace-Gadsden F, Simons HR, Matthews N, Labar AS, Hwang J, Wain J. Multi-locus sequence typing of enteroaggregative *Escherichia coli* isolates from Nigerian children uncovers multiple lineages. *PLoS One* 2010; **5**: e14093.
- Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2004; **2**: 123-40.
- Zamboni A, Fabbriotti SH, Fagundes-Neto U, Scaletsky IC. Enteroaggregative *Escherichia coli* virulence factors are found to be associated with infantile diarrhea in Brazil. *J Clin Microbiol* 2004; **42**: 1058-63.
- Vila J, Vargas M, Henderson IR, Gascón J, Nataro JP. Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhea strains. *J Infect Dis* 2000; **182**: 1780-83.
- Kaur P, Chakraborti A, Asea A. Enteroaggregative *Escherichia coli*: An Emerging Enteric Food Borne Pathogen. *Interdiscip Perspect Infect Dis* 2010; **2010**: 254159.
- Weintraub A. Enteroaggregative *Escherichia coli*: epidemiology, virulence and detection. *J Med Microbiol* 2007; **56**: 4-8.
- Guion CE, Ochoa TJ, Walker CM, Barletta F, Cleary TG. Detection of diarrheagenic *Escherichia coli* by use of melting-curve analysis and real-time multiplex PCR. *J Clin Microbiol* 2008; **46**: 1752-57.
- Jafari F, Shokrzadeh L, Hamidian M, Salmanzadeh-Ahrabi S, Zali MR. Acute diarrhea due to enteropathogenic bacteria in patients at hospitals in Tehran. *Jpn J Infect Dis* 2008; **61**: 269-73.
- Pourakbari B, Ferdosian F, Mahmoudi S, Teymuri M, Sabouni F, Heydari H, Ashtiani MT, Mamishi S. Increase resistant rates and ESBL production between *E. coli* isolates causing urinary tract infection in young patients from Iran. *Braz J Microbiol* 2012; **43**: 766-69.
- Morin N, Santiago AE, Ernst RK, Guillot SJ, Nataro JP. Characterization of the AggR regulon in enteroaggregative *Escherichia coli*. *Infect Immun* 2013; **81**: 122-32.
- Andrade JA, Freymüller E, Fagundes-Neto U. Pathophysiology of enteroaggregative *Escherichia coli* infection: an experimental model utilizing transmission electron microscopy. *Arq Gastroenterol* 2010; **47**: 306-12.

17. Hegde A, Ballal M, Shenoy S. Detection of diarrheagenic *Escherichia coli* by multiplex PCR. *Indian J Med Microbiol* 2012; **30**: 279-84.
18. Okhuysen PC, Dupont HL. Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. *J Infect Dis* 2010; **202**: 503-5.
19. Jafari A, Aslani M Mi, Bouzari S. Enteroaggregative *Escherichia coli*, a heterogenous, underestimated and under-diagnosed *E. coli* pathotype in Iran. *Gastroenterol Hepatol Bed Bench* 2013; **6**: 71-79.
20. Aslani MM, Alikhani MY, Zavari A, Yousefi R, Zamani AR. Characterization of enteroaggregative *Escherichia coli* (EAEC) clinical isolates and their antibiotic resistance pattern. *Int J Infect Dis* 2011; **15**: e136-39.
21. Salmanzadeh-Ahrabi S, Habibi E, Jaafari F, Zali MR. Molecular epidemiology of *Escherichia coli* diarrhoea in children in Tehran. *Ann Trop Med* 2005; **25**: 35-9.
22. Soleimani M, Morovvati A, Hosseini SZ, Zolfaghari MR. Design of an improved multiplex PCR method for diagnosis of enterohaemorrhagic *E.coli* and enteropathogenic *E.coli* pathotypes. *Gastroenterol Hepatol Bed Bench* 2012; **5**: 106-11.
23. Souza TB, Lozer DM, Kitagawa SM, Spano LC, Silva NP, Scaletsky IC. Real-time multiplex PCR assay and melting curve analysis for identifying diarrheagenic *Escherichia coli*. *J Clin Microbiol* 2013; **51**: 1031-33.