Molecular study on diarrheagenic *Escherichia coli* pathotypes isolated from under 5 years old children in southeast of Iran

Hesam Alizade1, Reza Ghanbarpour2, Mohammad Reza Aflatoonian3*

1Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Department of Microbiology, Sirjan Faculty of Medical Sciences, Kerman University of Medical Sciences, Kerman, Iran
2Molecular Microbiology Department, Faculty of Veterinary Medicine, Shahid Bahonar University, Zoonosis Research Committee of Kerman University of Medical Sciences, Kerman, Iran
3Leishmaniose Research Committee of Kerman University of Medical Sciences, Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran

**Objective**: To determine the phylogenetic groups and prevalence of diarrheagenic *Escherichia coli* (*E. coli*) genes from children less than five years of age with diarrhea in southeast of Iran.

**Methods**: A total of 142 *E. coli* isolates were isolated from diarrheic samples. The isolates were examined for detection of virulence determinants and their phylogenetic background by PCR technique.

**Results**: The *E. coli* isolates fall into four phylogenetic groups: A (40.14%), B1 (18.31%), B2 (16.90%) and D (24.65%). Eighty isolates were positive for at least one of the examined DEC genes. *E. coli* isolates were classified in enterotoxigenic *E. coli* (52 isolates), enteroaggregative *E. coli* (23), atypical enteropathogenic *E. coli* (9), enteroinvasive *E. coli* (2).

**Conclusions**: This study demonstrated the importance of enterotoxigenic *E. coli* and enteroaggregative *E. coli* pathotypes in the childhood diarrhea. An epidemiologic surveillance especially for DEC, would be useful in control and prevention of infectious diarrhea in children.

**ARTICLE INFO**

**Article history:**
Received 12 May 2014
Received in revised form 27 May 2014
Accepted 22 Aug 2014
Available online 28 Aug 2014

**Keywords:**
*Escherichia coli*
Diarrhea
Phylogenetic group

**1. Introduction**

Gastrointestinal infections due to pathogenic *Escherichia coli* (*E. coli*) are significant causes of morbidity and mortality in children, particularly in developing countries[1]. Clinical categories of *E. coli* comprise commensal, intestinal pathogenic and extra-intestinal pathogenic strains. Diarrheagenic *E. coli* (DEC) pathotypes include enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and diffusely adherent *E. coli*[2]. ETEC pathotype defined by the presence of plasmid–encoded enterotoxins, comprise thermostable toxin (*ST*) and the thermolabile toxin (*LT*). ETEC strains are the most common cause of childhood diarrhea among all *E. coli* pathotypes and the major cause of diarrhea in travelers to developing countries[3]. Several virulence factors of EAggEC associated with diarrhea in children. Most of the genes encoding these virulence factors are located in the pAA plasmid, such as probe CVD432 and transcriptional factor encoded by the *aggR* gene. The pAA plasmid also carries the *aap* gene, which secreted low–molecular weight protein that promotes dispersal of EAggEC on the intestinal mucosa and facilitates efficient colonization[4,5]. Outbreaks of EIEC diarrhea are usually food or water–borne. However, through person–to–person transmissions have also been reported[6]. EIEC strains are able to attack intestinal epithelial cells. The invasion plasmid antigen H
\(\text{ipaH}\) gene sequence is used for the diagnosis of EIEC [7,8]. EPEC strains express eaeA gene, which produce intimin, and bundle forming pili (bfpA) responsible for the attaching and effacing lesions of intestinal microvilli [3,9]. Shiga–toxin–producing \(E.\ coli\) or EHEC are principal emerging pathogens that cause food and water–borne diarrheal diseases in humans. All Shiga–toxin–producing \(E.\ coli\) strains possess stx1 and/or stx2 genes that produce two powerful cytotoxins, called Shiga toxin [10]. The eaeA gene of EHEC shares considerable homology with the bfpA gene of EPEC. Attaching and effacing \(E.\ coli\) strains (eaeA+) that harbor the bfpA gene are classified as typical EPEC and strains that do not possess bfpA gene are classified as atypical EPEC [11,12]. There are important regional differences in the prevalence of different categories of DEC in South and Southeast Asia [13].

Strains of the phylogenetic groups differ in their genotypic and phenotypic characteristics, comprising their antibiotic–resistance profiles, their ability to exploit different sugars sources and their growth rate temperature relationships. Phylogenetically, \(E.\ coli\) strains are divided upon amplification of chuA and yjaA genes and DNA fragment TSP64.C2. The patterns of amplicons assigned four strains are divided upon amplification of chuA and yjaA genes and DNA fragment TSP64.C2. The patterns of amplicons assigned four

2. Materials and methods

2.1. Sampling and bacteriological identification

One hundred and forty two \(E.\ coli\) isolates were obtained from diarrheal samples of children under five years old. Isolates were collected between 2010 and 2012 from children referring to the laboratories of Kerman Province, southeastern Iran. Samples were cultured on Mac Conkey agar and eosin methylene blue (Biolife Laboratories, Milano, Italy). Standard bacteriological methods were used to confirm the \(E.\ coli\) isolates. Isolates were stored in Luria–Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at –70°C for further analysis.

2.2. Reference strains

Five \(E.\ coli\) strains were used as positive controls: \(E.\ coli\) H10407 for ETEC (\(LT^+\), \(ST^+\)), \(E.\ coli\) 85b for EIEC (\(ipaH^+\)), \(E.\ coli\) O42 for EAggEC (probe CVD432+, aggR+ and aap+), \(E.\ coli\) Sakai for EHEC and atypical EPEC (stx1+, stx2+ and eaeA+) and \(E.\ coli\) ECOR62 for (chuA+, yjaA+ and Tspe4. C2+). \(E.\ coli\) strain MG1655 was used as a negative control for virulence genes. All the reference strains were from the bacterial collection of Microbiology Department of Ecole Nationale Vétérinaire Toulouse, France.

2.3. PCR protocol

DNA was extracted from \(E.\ coli\) isolates and reference strains by lysis method. All isolates were tested by multiplex PCR assay for the presence of the \(LT\), \(ST\) and \(ipaH\) genes by Aranda et al. [14], for stx1, stx2 and eaeA genes by China et al. and probe CVD432, agg, aap genes by Cerna et al. [15,16]. The phylogenetic groups (A, B1, B2, and D) of each \(E.\ coli\) isolate were carried out by triplex PCR method as described previously [17]. The primers used for detecting sequences encoding virulence genes and phylogenetic groups are described in Table 1.

| Gene or probe | Primer sequence (5'–3') | Product size (bp) | Reference |
|---------------|--------------------------|------------------|-----------|
| ETEC LT       | GCC GAC AGA TTA TAC CTT GC| 450              | [4]       |
|               | CGG TCT CTA TAT CTC TCT TT|                 |           |
| ETEC ST       | ATT TTT CTT TCT GTA TCG TCT T| 190              |           |
|               | CAC CCG GTA CAA GCA GGA TT|                 |           |
| EAggEC Probe CVD432 | CGG GAC AAA GAC CTT ACT AT| 600              | [16]      |
| aggR           | CTA ATG GTA CAA TGC TGC TA| 457              |           |
|               | AGA GTC CAT CTC TTT GAT AAG|                 |           |
| aap            | CTT GAG TAT CAG GCT GAA TG| 310              |           |
|               | AACCCA TTC GCT TAG AGC AC|                 |           |
| EHEC ipaH      | GCT CTC TGA GGG CTT TTC CCA TAC CCT GCT GC| 600              | [4]       |
| EHEC aggR      | GCC GAC TAA CCA CCC TCT GAG ACT AC|                 |           |
| EPEC & eaeA    | AGG GCT GGA AAA GAG ATG TG| 570              | [15]      |
| EPEC, aggR     | CCA TGC TCA CCA GAG GA| 388              |           |
| stx1           | AGA GGC ATG TTA CCG TGT G| 807              |           |
| stx2           | CAC ATG CTC TGT GAC TCT CTT|                 |           |
| Phylogroup yjaA| TGA ACT GTC AGG AGA CGC TG| 211              | [17]      |
|               | ATG GAG AAC GAC TGC TGC|                 |           |
|               | CCC GCC AAG AAA GAA TGA CCA|                 |           |

3. Results

3.1. Phylogenetic grouping

The triplex PCR assays for phyotyping of isolates revealed that isolates fall into four phylogenetic groups, whereas 40.14% (57 isolates) belonged to A, 18.31% (26 isolates) to B1, 16.90% (24 isolates) to B2 and 24.65% (35 isolates) to D phylogenetic groups.
3.2. Detection of DEC isolates

Multiplex PCR were performed to detect the main five categories of E. coli. PCR assays revealed that 80 isolates were positive for at least one of the examined DEC genes. Fifty-two (36.62%) isolates were positive for LT and/or ST genes. The ETEC pathotype coding genetic marker ST and LT were the most prevalent genes in the isolates, while were detected in 11.97% and 9.86% of isolates respectively. Among 52 isolates possess ETEC pathotype genes 21 isolates (41.17%) were positive for both LT and ST genes (Table 2). Overall 23 (16.20%) of the 142 E. coli isolates analyzed carried the EAggEC encoding genes, while probe CVD432 and aap genes were detected in 9.86% and 6.34% of isolates respectively.

None of the isolates were positive for aggR gene (Table 2). The atypical EPEC isolates distributed in phylo-groups B1 (2 isolates) and B2 (2 isolates) phylogenetic groups. The EIEC strains coding genetic marker ipaH belonged to D (2 isolates) phylogenetic group (Table 2).

Table 2
Distribution of pathotypes in phylogenetic groups from children less than five years old.

| DEC Gene | Total No. (%) | Phylo-group |
|----------|---------------|-------------|
|          | A  | B1  | B2  | D  |
| ETEC LT  | 14  | 9.86 | 5   | 35.71 | 2 | 14.29 |
| ST       | 17  | 11.97| 5   | 29.41 | 7 | 41.18 |
| ETEC LT/ST | 21  | 14.79| 17  | 80.96 | 2 | 9.02 |
| EAggEC Probe CVD432 | 14  | 9.86 | 2   | 22.22 | 5 | 56.56 |
| aap      | 9   | 6.34 | 2   | 22.22 | 2 | 22.22 |
| EIEC ipaH | 2   | 4.92 | 4   | 22.22 | 2 | 22.22 |
| EPEC eaeA | 6   | 4.25 | 2   | 22.22 | 7 | 77.78 |
| EHEC stx1 | 6   | 4.25 | 2   | 22.22 | 7 | 77.78 |
| EHEC stx2 | 6   | 4.25 | 2   | 22.22 | 7 | 77.78 |
| Total    | 86  | 60.56| 26  | 18.30 | 16 | 11.26 |

3.3. Distribution of DEC genes in phylo-groups

Among 142 E. coli isolates 56.34% (80 isolates) and 43.66% (62 isolates) were positive and negative for at least one of the examined DEC genes respectively, which distributed in four phylo-groups (Table 3). ETEC strains were present among the isolates from A (21 isolates), B1 (17 isolates), B2 (7 isolates) and D (7 isolates) phylogenetic groups. Fourteen LT positive isolates belonged to B1 (7 isolates), B2 (5 isolates) and D (2 isolates) phylogenetic groups, while 17 isolates possess ST gene segregated in phylogenetic group A (5 isolates), B1 (7 isolates) and D (5 isolates) phylogenetic groups. Phylotyping of LT/ST positive isolates showed that the isolates belonged to A (17 isolates), B1 (2 isolates) and B2 (2 isolates) phylo-groups. EAggEC strains encoding probe CVD432 fell into B2 (5 isolates) and D (9 isolates) phylogenetic groups. The aap positive isolates were distributed in A (2 isolates), B2 (2 isolates) and D (5 isolates) phylogenetic groups. The atypical EPEC isolates were segregated in A (2 isolates) and B2 (7 isolates) phylo-groups. The EIEC strains coding genetic marker ipaH were detected in 9.86% and 6.34% of isolates respectively. In the present study EAggEC pathotype encoding genes, while probe CVD432 and aap genes were detected in 9.86% and 6.34% of isolates respectively.

4. Discussion

DEC is recognized as an important cause of both outbreaks and sporadic cases throughout the world. There are at least six pathotypes of E. coli including ETEC, EAggEC, EIEC, EPEC, EHEC and diffusely adherent E. coli, which can cause intestinal infection in humans. Phylogenetic analysis of E. coli isolates showed that DEC strains were distributed among groups A, B1 and D and commensal strains in groups A and B1[14]. On the other hand, surveying the evolutionary origins of pathogenic E. coli is to determine the phylogeny distribution of the virulence genes[18]. DEC are second most common cause of diarrhea among children under five years old[3].

The results of the present study highlight the importance of ETEC as a cause of childhood diarrhea in the studied region of Kerman, Iran. ETEC is the major etiologic agents but under-recognized bacterial cause of either infantile diarrhea in all age groups in areas with poor sanitation. This pathotype is the most important cause of traveler’s diarrhea; the organism is regularly imported to the developed world[19,20]. According to the results ST+ and LT+ isolates were detected in 11.97% and 9.86% of isolates respectively. In the other parts of world, there were reports differences from prevalence of ETEC pathotype. In studies on capital of Iran (Tehran) and Nicaragua 6.73% and 20.5% of diarrheic isolates obtained from children were positive for ETEC pathotype respectively[21,22]. Perez et al. indicated that 7.69% of E. coli isolates possessed ETEC encoding sequences[3]. In this study PCR results of phylogenetic determination, showed that ETEC pathotype mostly fell into group A, followed by B1, B2 and D. Escobar–Paramo et al. indicated that ETEC strains were found in A and B1 phylogenetic groups[23]. In a study, distribution of ETEC strains in phylo–groups were B1, A and D[3]. In the current study EAggEC pathotype encoding genes were examined. According to the results, probe CVD432+ and aap+ isolates were detected in 9.86% and 6.34%
of isolates respectively. The EAggEC pathotype has been implicated in endemic diarrhea among children in both industrialized and resource–poor countries[24]. In Tanzania a study on EAggEC isolates obtained from children less than five years old showed that prevalence of aggR+, aap+ and astA+ isolates were 61.6%, 26.7% and 15.1% respectively[5]. In Romanian, 11.6% of diarrheic isolates were positive for EAggEC pathotype that segregated to A (19 isolates), B1 (2 isolates), B2 (5 isolates) and D (3 isolates) phylogenetic groups[25], whereas in the current study 16.20% of isolates were positive for EAggEC pathotype and belonged to A, B2 and D phylo–groups. Boisen et al. surveyed potential virulence factors among 121 EAggEC strains isolated as part of a case–control study of moderate to severe acute diarrhea among children[24]. Among examined isolates prevalence of aggR and aap genes were 69.40% and 71.90% respectively and belonged to four phylogenetic groups A, B1, B2 and D. In the current study among 142 isolates nine eaeA+ isolates were detected which considered as atypical EPEC pathotype. Strains of EPEC are a well–known cause of diarrhoea particularly in infants and young children in less developed countries[26]. The results of an investigation on children with and without diarrhea in three Iranian Provinces, Tehran, Ilam and Mazandaran as a reservoir for intimin gene positive E. coli types showed that 40.5% and 20.0% of children with and without diarrhea harbored eaeA gene respectively[12]. On another study on 1610 E. coli isolates from patient age ranged from a few days to 98 years, 8.9% isolates were positive for EPEC and 4.8% positive for EAggEC pathotypes and 17 isolates were positive for both pathogens[27]. Phylogenetic analysis of DEC showed that EPEC strains were clustered mostly in groups B1, B2 and E[23]. The EIEC coding genetic marker (ipaH+) was a low frequency gene in the diarrheic isolates (4.92%). Similar reports showed that two isolates of the E. coli isolated from diarrheic children were positive for ipaH gene[22,28]. EIEC outbreaks are usually food or water borne; however, person–to–person transmission has also been reported[6]. This pathotype is extremely rare in southeast of Asia[8]. In a study, presence of the invasion–associated locus (ial) of the invasion plasmid was reported in 5% of children under two years old[29]. In Costa Rica, distribution of EIEC pathotype in each phylo–group indicated that isolates fell into A, B1 and D groups, whereas according to the results ipaH gene belonged to D phylogenetic group[3]. Phylogenetic analyses have shown that DEC strains fall into A, B1 and D phylo–groups[2]. None of the isolates possessed stx1 and stx2 genes and were not categorized as EHEC. This pathotype cannot be considered a main cause of childhood diarrhea in this region. These results are in accordance with the previous studies which were done on Thailand and Myanmar[30,31].

In conclusion ETEC and EAggEC were recovered at high rates from children with diarrhea, indicating a wide spread of these pathotypes in the study population. It is maybe that the proportion of E. coli pathotypes difference according to the geographic region. The PCR assay can facilitate epidemiologic surveillance of DEC contamination. It is may also be used in epidemiologic surveillance of water for human consumption and food samples for E. coli contamination. Moreover, these contaminations can be transmitted from adults to children. Detection of epidemiological information may contribute to the prevention, including vaccines and control of infectious diarrhea in children.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by a grant (No: 111.IDT.91) from Research Center for Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran. The authors are thankful to Dr. Eric Oswald (Ecole Nationale Vétérinaire Toulouse, France) for providing the reference strains.

References

[1] Franiczek R, Sobieszczanska B, Turniak M, Kasprzykowska U, Krzyzanowska B, Jermakow K, et al. ESBL–producing Escherichia coli isolated from children with acute diarrhea–antimicrobial susceptibility, adherence patterns and phylogenetic background. Adv Clin Exp Med 2012; 21: 187–192.
[2] Mokracka J, Koczura R, Jablonska L, Kaznowski A. Phylogenetic groups, virulence genes and quinolone resistance of integron–bearing Escherichia coli strains isolated from a wastewater treatment plant. Antonie Leeuwenhoek 2011; 99: 817–824.
[3] Perez C, Gomez–Duarte OG, Arias ML. Diarrheagenic Escherichia coli in children from Costa Rica. Am J Trop Med Hyg 2010; 83: 292–297.
[4] Aranda KRS, 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: 5849–5853.
[5] Mendez–Arancibia E, Vargas M, Soto S, Ruiz J, Kahigwa E, Schellenberg D, et al. Prevalence of different virulence factors and biofilm production in enterohaemorrhagic Escherichia coli isolates causing diarrhea in children in Ifakara (Tanzania). Am J Trop Med Hyg 2008; 78: 985–989.
Ahmed AM, Miyoshi S, Shinoda S, Shimamoto T. Molecular characterization of a multidrug-resistant strain of enteroinvasive *Escherichia coli* O164 isolated in Japan. *J Med Microbiol* 2005; 54: 273–278.

Liebchen A, Benz I, Mellmann A, Karch H, Gomes TAT, Yamamoto D, et al. Characterization of *Escherichia coli* strains isolated from patients with diarrhea in Sao Paulo, Brazil: identification of intermediate virulence factor profiles by multiplex PCR. *J Clin Microbiol* 2011; 49: 2274–2278.

Samorsnuk S, Chaicumpa W, van Seidlein L, Clemens JD, Sethabutr O. Using real time PCR to detect shigellosis: *ipadH* detection in Kaeng-Khoi District, Saraburi Province, Thailand. *Thammasat Int J Sci Technol* 2007; 12: 52–57.

Sobieszczanska B, Kasprzykowska U, Turniak M, Maciejewski A, Ahmed AM, Miyoshi S, Shinoda S, Shimamoto T. Molecular characterization of a multidrug-resistant strain of enteroinvasive *Escherichia coli* O164 isolated in Japan. *J Med Microbiol* 2005; 54: 273–278.

| Page | Content |
|------|---------|
| 6 | Ahmed AM, Miyoshi S, Shinoda S, Shimamoto T. Molecular characterization of a multidrug-resistant strain of enteroinvasive *Escherichia coli* O164 isolated in Japan. *J Med Microbiol* 2005; 54: 273–278. |
| 7 | Liebchen A, Benz I, Mellmann A, Karch H, Gomes TAT, Yamamoto D, et al. Characterization of *Escherichia coli* strains isolated from patients with diarrhea in Sao Paulo, Brazil: identification of intermediate virulence factor profiles by multiplex PCR. *J Clin Microbiol* 2011; 49: 2274–2278. |
| 8 | Samorsnuk S, Chaicumpa W, van Seidlein L, Clemens JD, Sethabutr O. Using real time PCR to detect shigellosis: *ipadH* detection in Kaeng-Khoi District, Saraburi Province, Thailand. *Thammasat Int J Sci Technol* 2007; 12: 52–57. |

...