Prevalence and virulence characteristics of enteroaggregative Escherichia coli in a case–control study among patients from Iran

Saeed Bafandeh, Fakhri Haghi and Habib Zeighami

Enteroaggregative Escherichia coli (EAEC) is an important agent of diarrhoeal diseases worldwide. The role of EAEC virulence factors in the clinical outcome of infection is not completely defined. This case–control study investigated the prevalence of EAEC, its virulence genes and the antimicrobial resistance profile of adult patients with and without diarrhoea attending three different hospitals in Zanjan, Iran. A total of 550 individual stool specimens (350 from diarrhoeal patients and 200 from patients without diarrhoea) were collected. One hundred and forty-one EAEC isolates were identified by a HEp-2 cell assay and PCR. EAEC isolates were detected with slightly higher frequency in patients with (27.7 %) than in patients without (22 %) diarrhoea (P < 0.05). The EAEC genes aggR, aap and pet were identified more frequently in case patients compared with controls (P < 0.05). Many of the EAEC isolates from the diarrhoeal patients had two or more virulence genes compared with those without diarrhoea (P < 0.05). EAEC isolates exhibited high-level resistance to amoxicillin (82.3 %), co-amoxiclav (78 %), aztreonam (73.8 %), tetracycline (66.6 %) and ceftazidime (63.8 %). In addition, 53.2 % of isolates were resistant to at least three different classes of antimicrobial agents and were considered to be multidrug resistant. These results indicate a high prevalence and heterogeneity of gene profiles of EAEC in diarrhoeal and control patients, and suggest that the presence of aggR, aap and pet, the number of genes present and the antimicrobial resistance profile may be markers for more-virulent EAEC isolates.

INTRODUCTION

Diarrhoeal diseases are a leading cause of childhood morbidity and mortality, accounting for approximately 2.5 million deaths annually worldwide (Azevedo Feitosa Ferro et al., 2012; Haghi et al., 2014). Diarrhoeagenic Escherichia coli are the most common bacterial pathogens implicated in diarrhoea and represent a major public health problem in developing countries (Tobias & Vutukuru, 2012).

Enteroaggregative Escherichia coli (EAEC) are a diverse subgroup of diarrhoeagenic E. coli and have emerged as an important pathogen in traveller’s diarrhoea, diarrhoea among children and immunocompromised patients (Khoshvaght et al., 2014). Whilst a growing number of studies have supported the association of EAEC with persistent diarrhoea, in many others it appears to cause sub-clinical infection or only intestinal colonization (Lima et al., 2013). EAEC has also been associated with chronic intestinal inflammation, leading to childhood malnutrition and growth impairment (Kaur et al., 2010; Lima et al., 2013).

The identification of EAEC depends on the characteristic aggregative adherence (AA) to HEp-2 cells in a ‘stacked-brick’-like pattern (Cennimo et al., 2009; Kaur et al., 2010). Several attempts have been made to develop a molecular biological assay for the rapid and inexpensive identification of EAEC. A cryptic DNA fragment sequence known as ‘CVD432’ or the AA probe, encoding an ABC transporter system, has been used as an EAEC molecular marker in epidemiological studies (Kaur et al., 2010; Khoshvaght et al., 2014). Some epidemiological studies have suggested that CVD432-positive strains, which are predicted to carry the AggR regulon, are the true EAEC pathogens, termed ‘typical EAEC’ (Kaur et al., 2010).

The AA phenotype in EAEC strains is associated with the presence of a high-molecular-mass plasmid (pAA) that encodes many virulence genes, including an anti-aggregation protein transporter (aat; previously referred to as CVD432 or the AA probe), enteroaggregative heat-stable toxin (astA), aggregative adherence fimbria I (aggA),

Abbreviations: AA, aggregative adherence; EAEC, enteroaggregative Escherichia coli; MDR, multidrug resistant
aggregative adherence fimbria II (aafA), dispersin secretory protein (aap), plasmid encoded toxin (pet) and transcriptional activator (aggR). E. coli α-haemolysin (Hly) and cytotoxic distending toxin are other putative virulence factors detected in some EAEC strains (Regua-Mangia et al., 2009; Scavia et al., 2008).

Although EAEC is considered an emerging pathogen, not all strains are associated with diarrhoeal disease. EAEC strains are a heterogeneous group with regard to the presence of putative determinants. The role and prevalence of these factors have been found to vary by location, making the precise diagnosis of EAEC infections problematic (Regua-Mangia et al., 2009). Recent work has focused on the genetics of EAEC responsible for pathogenesis and virulence.

The continuous emergence of resistance to antimicrobial agents among the prevalent pathogens is the most dangerous threat for the treatment of infectious diseases (Aslani et al., 2011). Compared with other diarrhoeagenic E. coli, EAEC was found to be highly resistant to many commonly used antimicrobial agents (Mendez Arancibia et al., 2009). Regular surveillance of antibiotic resistance provides information for antibiotic therapy and resistance control (Aslani et al., 2011).

The objectives of the present case–control study were to investigate the prevalence of EAEC and its virulence genes among patients with and without diarrhoea, and to determine the antimicrobial resistance profiles of these isolates.

**METHODS**

**Patients and bacterial isolation.** In this case–control study, a total of 550 adult patients participated voluntarily, comprising 350 consecutive patients with and 200 without diarrhoea attending three different hospitals in Zanjan, Iran. Patients were enrolled in the study if they had diarrhoea and had not taken any antimicrobial agent in the week preceding sampling. Diarrhoea was characterized by the occurrence of three or more loose, liquid or watery stools or at least one bloody loose stool in a 24 h period. Control subjects were adults with no history of diarrhoea or antibiotic therapy for at least 1 month. Fresh stool samples collected in Cary–Blair transport medium were cultured on MacConkey agar (Merck) and one colony per sample was identified by conventional biochemical tests. Verified isolates of E. coli were preserved at −70 °C in trypticase soy broth (Merck) containing 20 % (v/v) glycerol for further analysis.

**Identification of EAEC isolates.** E. coli isolates (one isolate per patient) were screened for EAEC by PCR targeting the pCVD432 gene (primers are shown in Table 1). All pCVD432-positive isolates were examined for HEp-2 cell line adherence as described by Donnenberg & Nataro (1995). The reference strains EAEC 042 and non-pathogenic E. coli ATCC 25922 served as positive and negative controls, respectively. An isolate was interpreted as being EAEC if it showed the characteristic ‘stacked-brick’ AA on co-culture with HEp-2 cells.

**Detection of EAEC virulence-related genes.** The presence of the EAEC plasmid-borne virulence markers aggR, aggA, aap, and pet was assessed using the primers listed in Table 1. DNA from EAEC isolates was purified using a ReadyAmp DNA Purification System (Promega). Single PCR was performed using DreamTaq PCR Master Mix (Fermentas), which contains Taq polymerase, dNTPs, MgCl2 and the appropriate buffer. Each PCR tube contained 25 μl reaction mixture composed of 12.5 μl Master Mix, 2.5 μl each forward and reverse primer solution (at a final concentration of 200 nM), 1.5 μl DNA and nuclease-free water to complete the final volume. PCR was performed using a Gene Atlas 322 System (ASTEC). Amplification involved an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation (94 °C, 1 min), annealing (55 °C for 1 min for pCVD432, astA, aap and aggR; 52 °C for 1 min for pet; 48 °C for 1 min for aggA; 50 °C for 45 s for aggA) and extension at 72 °C for 1 min, with a final extension step at 72 °C for 8 min. The amplified DNA was separated by submarine gel electrophoresis on 1.5 % agarose, stained with ethidium bromide and visualized under UV transillumination. The following EAEC strains were used as positive controls: JM221 (aggA) and 042 (aatA, aggR, aap, pet, astA, aafA).

**Antimicrobial susceptibility testing.** The susceptibility of isolates to the following antibiotics was examined using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines: amoxicillin (25 μg), aztreonam (30 μg), ampicillin (10 μg), gentamicin (15 μg), chloramphenicol (30 μg), tetracycline (30 μg), trimethoprim-sulphamethoxazole (25 μg), and ceftazidime (30 μg).

### Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCVD432-F</td>
<td>CTGGGGAAGGACGTATCAT</td>
<td>630</td>
<td>(Aslani et al., 2011)</td>
</tr>
<tr>
<td>pCVD432-R</td>
<td>AATGTATAGAAATCCGCTGTT</td>
<td>630</td>
<td>(Aslani et al., 2011)</td>
</tr>
<tr>
<td>aggR-F</td>
<td>GTATACACAAAAAGAAAGGACG</td>
<td>254</td>
<td>(Aslani et al., 2011)</td>
</tr>
<tr>
<td>aggR-R</td>
<td>ACAGAATCGTCAGCATCAGC</td>
<td>457</td>
<td>(Aslani et al., 2011)</td>
</tr>
<tr>
<td>aggA-F</td>
<td>TTAGTCTTTCTATCTAGGG</td>
<td>242</td>
<td>(Aslani et al., 2011)</td>
</tr>
<tr>
<td>aggA-R</td>
<td>AAATTTAATTCGGGCAATG</td>
<td>310</td>
<td>(Aslani et al., 2011)</td>
</tr>
<tr>
<td>aafA-F</td>
<td>TGGAGATTGGTACCTTTATAT</td>
<td>832</td>
<td>(Huang et al., 2007)</td>
</tr>
<tr>
<td>aafA-R</td>
<td>ATTGACCGTGATTGGCTGCC</td>
<td>111</td>
<td>(Aslani et al., 2011; Huang et al., 2007)</td>
</tr>
</tbody>
</table>
amikacin (30 µg), cefotaxime (30 µg), cefoxitine (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), co-amoxiclav (30 µg), co-trimoxazole (25 µg), cefepime (10 µg), gentamicin (10 µg), imipenem (10 µg) and tetracycline (30 µg) (Mast Group). Isolates shown to be resistant to at least three different classes of antimicrobial agents were determined to be multidrug resistant (MDR). E. coli ATCC 25922 was used as a control for antibiotic resistance.

Statistical analysis. The data were analysed with SPSS version 17.0 software (SPSS). A χ² test was used to determine the statistical significance of the data. A P value of ≤ 0.05 was considered significant.

RESULTS

Detection and characterization of EAEC from clinical stool samples

A total of 350 patients with diarrhoea and 200 control patients without diarrhoea were studied. Among these patients, 191 (34.7 %) were younger than 30 years, 214 (38.9 %) were 30–45 years and 145 (26.4 %) were ≥ 45 years. The sex distribution was 318 (57.8 %) male and 232 (42.2 %) female.

Among the 350 consecutive case patients presenting with diarrhoea, 27.7 % (n=97) were identified as having EAEC in their stool samples. EAEC was also isolated from 22.0 % (n=44) of patients without diarrhoea. All pCVD432-positive isolates (141 isolates) were confirmed as EAEC by the HEp-2 cell line adherence assay. EAEC isolates were identified with slightly higher frequency in diarrhoeal patients than in the control group (P≥0.05).

All EAEC isolates were tested by PCR to detect the virulence-related genes aggR, aafA, aap, pet and astA. Of 141 isolates harbouring the pCVD432 gene, 96 (77 isolates of diarrhoeal patients and 19 isolates of control group; P≤0.05) had the aggR gene; these were classified as typical EAEC. For all EAEC isolates in the case and control patients, the most frequent virulence genes identified were astA (82.2 %), aap (76.6 %), aggR (68.1 %) and aafA (46.8 %) (Table 2). The virulence genes identified more frequently among the EAEC isolates derived from the diarrhoeal patients compared with non-diarrhoea group were aggR, aap and pet (P≤0.05). Several different combinations of the virulence genes were found among the EAEC isolates. Table 3 shows that many of the EAEC isolates in the diarrhoea group had two or more virulence genes compared with isolates in the non-diarrhoea group (P≤0.05). The most frequent combination in the diarrhoea group was astA-aap-aggR (29.9 %), followed by astA-aap-aggR-aafA (16.5 %) and astA-pet-aap-aggR-aafA (11.3 %). EAEC isolates with the combination of aggR, aap and astA, with or without additional factors, were particularly strongly associated with diarrhoea (P≤0.05). Many of the EAEC isolates (49.9 %) in the control group had only one virulence gene (astA, 20.4 %; aap, 18.2 %; aafA, 11.3 %). EAEC isolates negative for all studied virulence genes were identified more frequently from the non-diarrhoea group (P≤0.05).

Susceptibility to antimicrobial agents

The antimicrobial resistance patterns of the isolates are presented in Table 4. Overall, 130 (92.2 %) EAEC isolates were resistant to one or more of the 13 tested antimicrobial agents, with the highest resistance found against amoxicillin (82.3 %), co-amoxiclav (78.0 %), aztreonam (73.8 %), tetracycline (66.6 %) and ceftazidime (63.8 %) (Table 2). Imipenem showed the highest activity against isolates and only 3.5 % of isolates were imipenem resistant. A total of 75 (53.2 %) isolates were resistant to at least three different classes of antimicrobial agents and considered

<table>
<thead>
<tr>
<th>Gene</th>
<th>EAEC diarrhoea group (n=97)</th>
<th>EAEC non-diarrhoea group (n=44)</th>
<th>Total (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aggR</td>
<td>77 (79.4)</td>
<td>19 (43.2)</td>
<td>96 (68.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aafA</td>
<td>45 (46.4)</td>
<td>21 (47.7)</td>
<td>66 (46.8)</td>
<td>0.884</td>
</tr>
<tr>
<td>aggA</td>
<td>5 (5.1)</td>
<td>3 (6.8)</td>
<td>8 (5.6)</td>
<td>0.695</td>
</tr>
<tr>
<td>astA</td>
<td>81 (83.5)</td>
<td>35 (79.5)</td>
<td>116 (82.2)</td>
<td>0.572</td>
</tr>
<tr>
<td>pet</td>
<td>24 (24.7)</td>
<td>2 (4.5)</td>
<td>26 (18.4)</td>
<td>0.004</td>
</tr>
<tr>
<td>aap</td>
<td>86 (88.6)</td>
<td>22 (50)</td>
<td>108 (76.6)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
DISCUSSION

EAEC are a heterogeneous group of E. coli that display variation in causing diarrhoeal illness, and their virulence-related factors are poorly understood (Dallman et al., 2014). An increasing number of studies support the association of EAEC with diarrhoea in populations in developing countries, most prominently in association with persistent diarrhoea. In several studies, culture of EAEC from the stool during the first few days of diarrhoea is predictive of a longer duration of illness. The association of EAEC with diarrhoea appears to vary geographically, and many studies have demonstrated the importance of EAEC in paediatric diarrhoea. In studies carried out in Vietnam and the USA, EAEC was isolated at higher prevalence in children with diarrhoea (11.6 and 4.5 %, respectively) than in controls (7.2 and 1.7 %, respectively) (Nataro et al., 2006; Nguyen et al., 2005; Usein et al., 2009). Our findings, however, were in contrast to these studies. According to our results, EAEC pathotype was detected with slightly higher frequency among adults with diarrhoea, but the association between EAEC and diarrhoea was not significant. The prevalence of this pathotype in diarrhoeal patients and the control group was 27.7 and 22 %, respectively. This lack of association with diarrhoea was observed in our previous study among diarrhoeal children (Haghi et al., 2014). In a study carried out in Nicaragua, EAEC strains were the most frequently isolated pathotype of E. coli; however, the isolation rate as a total was slightly higher in the healthy group than in the diarrhoea group (Vilchez et al., 2009). Among the few published studies in Iran, only Salmanzadeh-Ahrabi and co-workers reported the association of EAEC isolates with diarrhoea: they reported EAEC isolates in 24 % of children with diarrhoea and 8 % of controls (P ≤0.0001) (Jafari et al., 2013; Salmanzadeh-Ahrabi et al., 2005). Our results also support the fact that EAEC is a heterogeneous group of E. coli and not all strains are capable of causing diarrhoea. In agreement with previous reports (Lima et al., 2013), our isolates harboured a diverse range and combination of virulence-related genes. The genes encoding AggR, Aap and Pet were associated with diarrhoea. Other reports have not found a correlation between the pet gene and the occurrence of diarrhoeal disease (Huang et al., 2007; Pereira et al., 2007). However, in vitro studies have shown that this cytotoxin induces modification in the cytoskeleton followed by cell rounding and detachment of cell monolayers in culture (Betancourt-Sanchez & Navarro-García, 2009). The term ‘typical’ EAEC has been suggested to classify EAEC isolates harbouring the AggR gene and the occurrence of diarrhoeal disease (Cennimo et al., 2009). The term ‘typical’ EAEC has been suggested to classify EAEC isolates harbouring the AggR gene and the occurrence of diarrhoeal disease (Cennimo et al., 2009). Among the few published studies in Iran, only Salmanzadeh-Ahrabi and co-workers reported the association of EAEC isolates with diarrhoea: they reported EAEC isolates in 24 % of children with diarrhoea and 8 % of controls (P ≤0.0001) (Jafari et al., 2013; Salmanzadeh-Ahrabi et al., 2005). Our results also support the fact that EAEC is a heterogeneous group of E. coli and not all strains are capable of causing diarrhoea. In agreement with previous reports (Lima et al., 2013), our isolates harboured a diverse range and combination of virulence-related genes. The genes encoding AggR, Aap and Pet were associated with diarrhoea. Other reports have not found a correlation between the pet gene and the occurrence of diarrhoeal disease (Huang et al., 2007; Pereira et al., 2007). However, in vitro studies have shown that this cytotoxin induces modification in the cytoskeleton followed by cell rounding and detachment of cell monolayers in culture (Betancourt-Sanchez & Navarro-García, 2009). The term ‘typical’ EAEC has been suggested to classify EAEC isolates harbouring the AggR gene and the occurrence of diarrhoeal disease (Cennimo et al., 2009). The term ‘typical’ EAEC has been suggested to classify EAEC isolates harbouring the AggR gene and the occurrence of diarrhoeal disease (Cennimo et al., 2009).
more virulence genes compared with the non-diarrhoea group. The variation of EAEC virulence genes in our study confirms the genetic heterogeneity of this organism. As most of the virulence-related genes are encoded on the plasmid, this may help to explain the dynamic horizontal acquisition and loss of these genes (Lima et al., 2013).

Antimicrobial resistance among human pathogens has become a major public health problem worldwide. In a study carried out in Kolkata, India, the majority of EAEC isolates from diarrhoeal patients exhibited MDR including fluoroquinolone resistance (Kahali et al., 2004). Peruvian EAEC isolates were also MDR, especially to ampicillin, co-trimoxazole, tetracycline and nalidixic acid (Ochoa et al., 2009). According to our results, the EAEC isolates exhibited high-level resistance to various antibiotics including amoxicillin, co-amoxiclav, aztreonam, tetracycline and ceftazidime. The high incidence of antibiotic resistance found in this survey was most probably due to the widespread use of numerous antimicrobial agents such as amoxicillin in our country. Furthermore, the loss and gain of resistance genes by mobile genetic elements such as the pCVD plasmid is an important mechanism in the development of MDR isolates (Gündoğdu et al., 2011).

In conclusion, our study has demonstrated a similar prevalence of EAEC in diarrhoeal and healthy control adults in Zanjan, Iran. The genes encoding AggR, Aap and Pet appeared to be commonly associated with diarrhoea and may be good markers for EAEC diagnosis.

REFERENCES


