Molecular epidemiological characteristics of virulence factors on enteroaggregative E. coli

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Received 11 May 2005; received in revised form 22 September 2005; accepted 25 September 2005

First published online 11 October 2005

Edited by M. Schembri

Abstract

Escherichia coli with various types of adherence patterns to cultured epithelial cells have been described over the years as being associated with both acute and persistent diarrhea. Most enteroaggregative E. coli (EAEC) strains harbor a 60- to 65-MDa plasmid called pAA which has been shown to encode the aggregative adherence fimbriae AAF/I and AAF/II; the enterotoxin EAST1 and Pet, a serine protease which has been described as causing enterotoxic and cytotoxic effects. Another serine protease denominated Pic, encoded by a chromosomal gene displaying mucinolytic activity, serum resistance, and hemagglutination, has also been associated with EAEC strains. In this study, EAEC strains that isolated from the rectal swab of neonates at the neonatal intensive care unit of Pusan National University Hospital in 2003 were tested for the presence of the pAA using polymerase chain reaction (PCR) and DNA colony hybridization methods. To further characterize these EAEC strains, we used PCR to detect genes for proposed EAEC virulence factors and examined HeLa cell adherence assay, antimicrobial susceptibility test, serotyping, cytotoxicity test and epidemiological characteristics. EAEC isolates found were genotyped by random amplified polymorphic DNA and pulsed-field gel electrophoresis.

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Keywords: Enteroaggregative E. coli; Virulence factor; Adherence; PCR; RAPD; PFGE

1. Introduction

Escherichia coli infection is an important cause of illness and death in infants in developing countries. Diarrhea continues to be one of the most common causes of morbidity and mortality among infants and children [1]. Five distinct classes of diarrheagenic E. coli (DEC) are recognized as being associated with diarrheal disease. They are enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), enteropathogenic E. coli (EPEC), enterohemorrhagic E. coli (EHEC), and enteroaggregative E. coli (EAEC). Diffuse adhering E. coli (DAEC) may represent a sixth category, but this has not been clearly established [2]. Classification is based on known or putative virulence factors, clinical syndromes, or other characteristic markers, such as the adherence phenotype. Thus, the detection of DEC has focused increasingly on the identification of certain characteristics which themselves determine the virulence of these organisms.

Nataro et al. [3] first reported that some E. coli isolates from diarrhea patients had a capacity to adhere to HEp-2 cells and the surface of glass Petri dishes, and named them EAEC. The bacteria appear as...
“stacked brick” clumps which adhere to both the HEp-2 cells and the glass coverslip. The aggregative adherence of EAEC was due to the presence of aggregative adherence fimbriae (AAF), AAF/I and AAF/II, whose expression is positively controlled by the aggR gene [4]. AggR controls expression of adherence factors, a dispersin protein, and a large cluster of genes encoded on the EAEC chromosome [5]. A 1-kb fragment of pAA (pAA; aggregative adherence plasmid), referred to as the EAEC probe or CVD432, has been widely used for epidemiological studies [6]. The pAA plasmid encodes aggregative adherence fimbriae (AAF) I, II, and III [4,7]; the transcriptional activator AggR [4]; enteric-aggregative heat-stable enterotoxin 1 (EAST-1) [8]; a 104-kDa cytotoxin designated Pet [9]. In addition to the pAA plasmid, some EAEC strains express putative virulence factors that are encoded on the chromosome, including a 116-kDa secreted mucinase (Pic). Shigella enterotoxin 1 (ShET1) is encoded by the antisense strand of the pic gene [10]. Despite the variety of virulence factors found in EAEC strains, the essential features of EAEC pathogenesis are still not well understood. The PCR technique used here enables a more rapid diagnosis of EAEC than the other techniques currently available. However, the EAEC probe or the adherence test must still supplement the PCR to identify the disease-causing strains. Because one EAEC probe-positive strain was found to be negative in the cell culture assay, the typical aggregative pattern can only be determined by cell culture. Elias et al. [6] suggest the possibility that EAEC probe-positive strains comprise a distinct subcategory of EAEC that could be called typical EAEC. Therefore, the adherence test appears to have the highest level of specificity and is recommended to be used as a definitive confirmation test. Epidemiologic and clinical investigations in many settings should now include detection of typical EAEC.

This study examined E. coli strains from neonates with sporadic cases of diarrhea and those without diarrhea for the presence of the pAA. The strains possessing the plasmid were further analyzed for their adherence patterns and the presence of virulence markers associated with EAEC. The EAEC strains that colony-blot hybridized with the probe for pAA were also assayed HeLa cell adhesion test. In addition to biochemical and serological characteristics, cytotoxicity test was examined for the characterization of EAEC and epidemiological characteristics were analyzed.

2. Materials and methods

2.1. Bacterial strains

A total of 183 strains of E. coli were isolated from the rectal swab of neonates at the neonatal intensive care unit of Pusan National University Hospital in 2003. To identities of these isolates as E. coli were confirmed by different biochemical tests by standard procedure [11]. Initial screening for EAEC was performed by EAEC-PCR with the primers pCVD432/start (5′-CTG GCG AAA GAC TGT ATC ATA AT-3′) and pCVD432/stop (5′-CAA TGT ATA GAA ATC CGC TGT T-3′) (GenBank Accession No. X81423) [12]. A primer pair complementary to EAEC probe was designed for PCR amplification of a 630-bp region. The strains that hybridized with the probe for pAA were also assayed on HeLa cells.

2.2. DNA hybridization and determination of EAEC adherence to HeLa cells

Bacterial strains that gave equivocal or negative results in the EAEC-PCR assay were investigated for DNA corresponding to regions of the EAEC by colony blotting using the pCVD432 fragment probe and hybridization conditions described previously [12]. DNA probes were labeled by DIG DNA Labeling and Detection Kit (Roche Diagnostics GmbH Mannheim, Germany). EAEC isolates were subjected to a HeLa cell adherence test by the method described by Cravioto et al. [1].

2.3. PCR analysis

The pAA- strains were examined for the presence of the following virulence genes of EAEC by PCR: aggR (transcriptional activator for EAEC aggregative adherence fimbria I expression) [13], aggA (aggregative adherence fimbria I) [14], aafA (aggregative adherence fimbria II) [7], astA (EAEC heat-stable enterotoxin) [8], pet (EAEC plasmid-encoded heat-labile toxin) [9] and pic (serine protease precursor) [10] using the primers listed in Table 1. Each primer sequence and the conditions for PCR were obtained from the relevant references. PCR amplification was performed in a thermal cycler PTC-100 (MJ Research Inc., Waltham, MA.) and the PCR products were electrophoresed in 2% agarose gel.

2.4. Serotyping and antimicrobial susceptibility test

Nine EAEC strains were examined for O antigen with polyvalent and monovalent antisera (Denka Seiken Co., Ltd., Tokyo, Japan) by slide agglutination of heated (100 °C, 1 h) bacterial suspensions. The antimicrobial susceptibility test was examined by using disk diffusion method on a Mueller–Hinton agar to the following 9 antimicrobial agents: ampicillin, cefuroxime, cefoprazone, cephalothin, chloramphenicol, gentamicin, kanamycin, streptomycin and tetracycline (BBL, USA). Plates were inoculated and zone sizes were interpreted as described by the NCCLS.
2.5. Cytotoxicity assay and restriction fragment length polymorphism (RFLP)

Cytotoxic activity of EAEC was measured by LDH release from HeLa cells using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA). RFLP of the gene encoding astA was analysed for 9 strains of EAEC using PstI endonuclease (TaKaRa Biochemicals). The hybridization temperature was 42°C.

2.6. Random amplified polymorphic DNA (RAPD)

The primers for the RAPD method described by Band et al. [15] were used. PCR was performed in thermal cycler PTC-100. The following procedure was used: initial denaturation at 95°C for 4 min, followed by 45 cycles consisting of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C each. The amplification products were analyzed by electrophoresis in a 1.5% agarose gel. The gels were stained with ethidium bromide (0.5 µg.ml⁻¹) and photographed under UV illumination.

2.7. Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out by the procedure of Vial et al. [16]. Chromosomal DNA-embedded agarose plugs for pulsed-field gel electrophoresis (PFGE) analysis were prepared using the CHEF Bacterial DNA Plug Kit (Bio-Rad, Hercules, CA). The DNA was digested with restriction endonuclease XbaI (TaKaRa, Japan) and electrophoresed using the CHEF-DR III PFGE system (Bio-Rad) under the following conditions: Voltage, 5 V cm⁻¹; block 1, 22 h, with initial switching time of 5 s to final switching time of 35 s. A lambda DNA ladder with a size range of 48.5 kb to 1 Mb (Bio-Rad) was used as a size marker.

3. Results and discussion

Recently, EAEC have emerged as a significant cause of chronic infant or young children diarrhea in certain developing countries. EAEC strains possess the aggA gene that encodes the aggregative adherent fimbria I (AAF/I) protein [17], the aggR gene for transcriptional activation of AAF/I expression [4], and the astA gene that encodes the EAEC heat-stable enterotoxin I protein [8]. Since the characteristic aggregative adherence pattern of EAEC is associated with the presence of a large plasmid called pAA, DNA probes and PCR primers derived from this plasmid have been recommended as a screening method for EAEC in the clinical laboratory [12]. On the other hand, EAEC isolates have been examined for the presence of the following virulence genes. They are: asA (EAEC heat-stable enterotoxin) [18], aggA (structural gene) [19], aggR (transcriptional activator) [8,13], aggB, aggC [19], and aggD for EAEC aggregative adherence fimbria I (AAF/I), aafA for AAF/II [7], and pet for EAEC plasmid-encoded heat-labile toxin [9]. However, the pathogenic mechanisms of EAEC infection are not fully understood. Moreover, there appears to be significant heterogeneity of virulence among EAEC isolates. It is now firmly established that EAEC are very diverse, belonging to a broad range of serotypes, and not all strains carry the AAF/I, AAF/II and EAST1 genes [7,19]. Since only a few reports describe outbreaks of diarrhea involving EPEC and EAEC and the incidence of EAEC cases in patients with diarrhoea is low [20]. Therefore, we were interested in determining the incidence of EAEC in E. coli isolates from neonates, and assessed the prevalence of putative virulence factors in an attempt to identify their roles as enteric virulence factors. As a result, of the 183 E. coli strains isolated from neonates, 9 did test positive for pAA (Fig. 1) and the 9 pAA⁺ strains adhered to HeLa cells in a stacked-brick formation (Fig. 2) under the con-

**Table 1**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Properties of target</th>
<th>Primer sequence</th>
<th>Ref. (GenBank Accession No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aggA</td>
<td>AAF/I subunit</td>
<td>GCTAACGCTCGTGTAGAAAGACC</td>
<td>[14] (AY344586)</td>
</tr>
<tr>
<td>aafA</td>
<td>AAF/II subunit</td>
<td>GGAGTATCATTCTATATTGCC</td>
<td>[7] (AF012835)</td>
</tr>
<tr>
<td>aggR</td>
<td>Transcription of AAFs</td>
<td>GACAACCGCAACGCTGCGTG</td>
<td>[13] (Z32523)</td>
</tr>
<tr>
<td>astA</td>
<td>EAST-1 toxin</td>
<td>GCCATCAACACAGTATATCC</td>
<td>[8] (L11241)</td>
</tr>
<tr>
<td>pet</td>
<td>104-kDa cytotoxin</td>
<td>CGGAAATGAGCTGAAC</td>
<td>[9] (AF056581)</td>
</tr>
<tr>
<td>pic</td>
<td>Serine protease precursor</td>
<td>TCGCCTGTATATATATCTTGATTC</td>
<td>[10] (AF097644)</td>
</tr>
<tr>
<td>pCVD432 (pAA)</td>
<td>Aggregative adherence plasmid</td>
<td>CTGGCGAAAGACTGTATCAT</td>
<td>[12] (X81423)</td>
</tr>
</tbody>
</table>
ditions used in this study. The 9 EAEC strains were assayed by PCR to detect the genes for AAF/I, AAF/II, EAST1, Pet, and Pic. Table 2 shows the distribution of target genes among the EAEC isolates. Of the 9 EAEC strains isolated from neonates, we found that 9 strains (100%) possessed aggR and astA, 3 strains (33.3%) were aafA and pet positive but aggA negative. Pic (88.9%) protein was detected in most EAEC strains. Piva et al. [14] detected pic sequences significantly more frequently in EAEC probe-positive strains from children with diarrhea than from controls. Few studies have evaluated the prevalence of EAEC markers in EAEC probe-positive and probe-negative strains isolated from subjects in case-control studies. The present study provides evidence that some of the recognized pAA-encoded factors of EAEC (AAF/I, AAF/II, and AggR) are prevalent in EAEC probe-positive strains and that others (Pet, EAST-1, and ShET1/Pic) are found in EAEC strains. Recently, Jiang et al. [21] suggested that the aggA and aggR genes alone or in combination with other virulence factors (aafA or aap) might be used to identify pathogenic EAEC strains. However, in the present study, both AggR and EAST-1 virulence factors was the most common gene marker identified in EAEC

Fig. 1. Results of Southern hybridization analysis with pAA gene probe for EAEC strains.

Fig. 2. Patterns of HeLa cell adherence of EAEC strains. (A) Negative control (X400) and (B) aggregative adherence (X1000).

Table 2
Distribution of EAEC isolates by serotyping, antibiogram, RFLP, RAPD, PFGE and virulence factors

<table>
<thead>
<tr>
<th>Strains No.</th>
<th>Serotyping</th>
<th>Antibiogram</th>
<th>RFLPb</th>
<th>Detection of virulence factors</th>
<th>Adherence assayc</th>
<th>RAPD</th>
<th>PFGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OUT</td>
<td>AmClpCxmCfCgKSTe</td>
<td>2.5</td>
<td>– + + + + – +</td>
<td>AA</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>OUT</td>
<td>AmClpCfCg</td>
<td>2.5</td>
<td>– + + + + + +</td>
<td>AA</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>O8</td>
<td>AmClf</td>
<td>2.5</td>
<td>– + – – + – –</td>
<td>AA</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>O86a</td>
<td>AmCxmCfTe</td>
<td>2.5</td>
<td>– + – – + – –</td>
<td>AA</td>
<td>C</td>
<td>B</td>
</tr>
<tr>
<td>5</td>
<td>O86a</td>
<td>AmClpCxmCfGmTe</td>
<td>2.5</td>
<td>– + + + + + +</td>
<td>AA</td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>6</td>
<td>OUT</td>
<td>AmClpCxmCfGmSTe</td>
<td>2.5</td>
<td>– + – – – – –</td>
<td>AA</td>
<td>E</td>
<td>D</td>
</tr>
<tr>
<td>7</td>
<td>O25</td>
<td>AmClpCxmCfGmKSTe</td>
<td>2.5</td>
<td>– + – – + – –</td>
<td>AA</td>
<td>F</td>
<td>D</td>
</tr>
<tr>
<td>8</td>
<td>O25</td>
<td>AmClpCxmCfGmTe</td>
<td>2.5</td>
<td>– + – – + – –</td>
<td>AA</td>
<td>F</td>
<td>D</td>
</tr>
<tr>
<td>9</td>
<td>O25</td>
<td>AmClpCxmCfGm</td>
<td>2.5</td>
<td>– + – – + + ±</td>
<td>AA</td>
<td>F</td>
<td>E</td>
</tr>
</tbody>
</table>

a Ampicillin (Am), Cefoperazone (Cfp), Cefuroxime (Cxm), Cephalothin (Cl), Chloramphenicol (C), Gentamicin (Gm), Kanamycin (K), Streptomycin (S) and Tetracycline (Te).

b Length (kb) of PstI fragment(s) carrying the astA gene.

c Aggregative adherence (AA).
probe-positive strains and the Pic virulence marker was found at similar frequencies in EAEC strains.

Rich et al. [19] characterized 40 EAEC strains adhering to HEp-2 cells, and found that only 45% of the isolates harbored the EAST1-encoding genes as detected by PCR and 35% (14/40) generated positive results to the PCR primers specific for aggC. Czeczulin et al. [7] found that for the EAEC strains from various epidemiologic studies around the world, only 31% were detected as AAF/I positive and 12% were with AAF/II-encoding genes. Savarino et al. [18] found that 47% of the EAEC strains, 100% of the E. coli O157 strains, 41% of the ETEC strains and 22% of the EPEC strains they tested, had the EAST1 gene as assayed by DNA probe hybridization. EAEC has been recognized as DEC, since epidemiological information from case-control studies and outbreaks due to the organisms has accumulate and supported its significance [20,22]. Three serotypes (O8, O86a and O25) of 6 (66.7%) of the 6 isolates were assigned into EAEC in this study irrespective of whether their O antigens fitted into the scheme of EPEC. These serotypes have been reported to include not only EPEC but also EAEC or unusual EHEC [23]. And the other 3 isolates were not classified. The antibiotic susceptibility for EAEC was summarized in Table 2. All strains were resistant to ampicillin and cephalexin. Most isolates showed resistance to gentamicin (77.8%), cephaloridine (77.8%), cefuroxime (77.8%) and tetracycline (66.7%), respectively. They were resistant to at least four antibiotics except for one.

RFLP is useful for characterization of several organisms of medical importance. However, depending on the restriction enzyme used, the interpretation of RFLP patterns can be rather difficult because the multitude of chromosomal fragments present and subtle differences may not be observed. The PstI restriction fragment pattern of astA was grouped into one type (2.5 kb), respectively. A strong association between the restriction fragment patterns of astA was observed with EAEC strains. The chromosomal DNA fingerprinting is considered useful for the epidemiological study. Especially, RAPD and PFGE have traditionally been used for gene mapping and medical epidemiology. Genetic maps consisting of RAPD markers can be obtained more efficiently, and with greater marker density, than by RFLP methods [24]. So we evaluated the RAPD and PFGE techniques for studying E. coli molecular epidemiology. In this study, the RAPD technique revealed that AP-PCR profiles were 6 different types (Fig. 3). These patterns were compared with 5 different PFGE types. The typing results are summarized in Table 2. Our results indicated that RAPD is useful as a tool in investigations of microbial outbreaks or to supplement serotyping and PFGE of EAEC.

In conclusion, our data reinforce the heterogeneity of EAEC strains with regard to both pAA plasmid-borne

![Fig. 3. The results of 1.5% agarose gel electrophoresis for the amplicons of RAPD analysis. Lane M: phage λ DNA digested with HindIII (molecular mass marker); lane N: phage ΦX174 DNA digested with HaeIII (molecular mass marker).](https://academic.oup.com/femsle/article-abstract/253/2/215/506474)
and chromosomal factors. 9 EAEC strains from neonates had at least two virulence-association genes. This frequency was quite comparable to the previous studies performed in developing countries, and the number of reports describing outbreaks due to EAEC is increasing [22,25]. In South Korea, there are a few reports describing outbreaks of diarrhea involving EPEC and EAEC. Based on our cases and the reports previously mentioned, the contribution of EAEC to the human disease might be more significant than is currently appreciated. Furthermore, our data suggest that aggR can be used as an important genetic marker for EAEC probe-positive strains and the RAPD and PFGE techniques could be efficient tools in epidemiological studies of EAEC strains.

References