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Original Article

Prevalence of enteroaggregative *Escherichia coli* among children with gastroenteritis in the northwest of Iran

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Abstract

Introduction: To investigate the prevalence of enteroaggregative *Escherichia coli* (EAEC) in children < 10 years with a diagnosis of gastroenteritis by multiplex polymerase chain reaction (MPCR).

Methods: A total of 303 diarrheal stool samples from patients admitted to Tabriz children's hospital (the referral center in East Azerbaijan province of Iran) with diagnosis of gastroenteritis were enrolled in cross-sectional study for detection of three virulence genes using MPCR.

Results: EAEC infection was found in 55 cases (18.2%). aspU gene was the most frequently detected gene (51 of 55). Of 55 EAEC 27 (49.1%) had only aspU gene, 4 (7.3%) just pCVD432 gene and 24 (43.6%) samples had both genes (aspU and pCVD432) simultaneously. There was no sample harboring aggR gene. Prevalence of EAEC among girls and boys were 14.6% (18/123) and 20.5% (37/180), respectively. Prevalence of EAEC according to the age group was 17% for 0-5 years (42 of 247) and 23.2% for 5-10 years (13 of 56). There was no significant association between prevalence of EAEC and the age groups and also the gender of the patients (P > 0.05).

Conclusion: The present study revealed the high prevalence of EAEC in children with diarrhea in this region that should be more considered in preventing, diagnosis and treatment strategies. We conclude that using multiple virulence genes simultaneously for detection of this strain is necessary to gain reliable results with pointing to aspU as preferred gene for detecting EAEC when diagnosis based on the presence of one virulence marker. To the best of our knowledge, this is the first report on the prevalence of EAEC strains in the northwest of Iran.

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Introduction

The association of enteroaggregative *Escherichia coli* (EAEC) with diarrhea in children has been documented in different studies in industrially developing and developed countries.[1-3] EAEC was first described by Nataro et al.[4] in 1987 in a child with acute diarrhea in Lima, Peru. Since that, sporadic and outbreaks of acute and persistent diarrhea in different parts of the world (developing and developed countries) have been reported.[5-10]

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The known virulence traits of this diarrheagenic pathogen are represented by the flowing proteins; A 60 MDa plasmid encoded proteins aggregative adherence (AA) factor (I, II, III, IV), ST-like enterotoxin (EAST), an anti-aggregation protein transporter (CVD432), dispersin secretory protein (aap; aspU) and transcriptional activator AggR (aggR).[11,12]

Although the pathogenesis of EAEC infection is not fully understood but it has been reported that pathogenesis of this group of enteropathogens can be result of ability to adhere to intestinal cells, produce enterotoxins and cytotoxins, and induce inflammation.[13] A large-scale outbreak of diarrhea and hemolytic uremic syndrome (HUS) in northern Germany in 2011 caused by shiga toxin-producing EAEC revealed the alarming capability of this pathogen to genetically recombine resulting in a phenotype of devastating proportions.[14]

Importantly an emerging concept is the notion that certain enteric pathogens, including EAEC, cause not only acute and persistent diarrheal disease but can also persist in the human intestine subclinically, including chronic inflammation in the absence of dysentery. The chronic disruption in gut function by persistent infection has been linked to malnutrition and decreased physical and intellectual in children.[15]

Hep-2 cell line culture is the gold standard way for identification of EAEC strains because of their defined AA pattern in a so-called "stacked-brick" aggregation formation.[4] The adhesion method because of its difficulties to perform and needs of the experienced person to interpret the results has been increasingly supplemented by polymerase chain reaction (PCR) methods based on detecting virulence factors.[14] There is no information about prevalence of this group of enteropathogens in the northwest of Iran and because of inability of traditional and usual laboratory methods in differentiation of these pathogenic strains from the commensal kinds, the importance of these enteropathogenic agents are often overlooked.

The objectives of this study were: 1- To determine the prevalence of EAEC strains in children with gastroenteritis admitted to Tabriz children's hospital, Iran (the referral health care center for pediatrics), 2- Using three known virulence genes for identification of these diarrheagenic strains simultaneously by multiplex PCR (MPCR).

Methods

In the present study, 303 diarrheal samples from children, < 10 years of old admitted to Tabriz Children's Educational-Health Care Center-the only children's referral hospital in northwest of Iran-with diagnosis of gastroenteritis and presenting negative for parasitological agents of diarrhea were collected over 9 months of period (June-February 2013).

Gastroenteritis was defined as follows: All children with diarrhea that accompanied at least one of the symptoms such as vomiting, abdominal pain, and nausea. Diarrhea was defined as follows: Passing loose or watery stools at least 3 times in 24 hour or a loose bloody stool at least once in the past 24 hour.

Diarrheal samples from newborns were taken with rectal swabs and transported to the laboratory in Cary-Blair transport medium. In the cases of the younger children, the samples were collected in a caped container and transported to the microbiology laboratory. All samples after being confirmed negative for parasitological agents were cultured directly on McConkey and xylose lysine decarboxylase agar mediums and incubated at 37 °C for 24 hour. After the incubation period grown colonies were subjected to standard biochemical tests (triple sugar iron, sulfide-indole-motility, methyl red/Voges-Proskauer, citrate, lysine iron agar and urease) for differential identification of enterobacteriaceae strains. After identification, *E. coli* strains were subcultured in 20% of glycerolin trypticase soya broth (Oxoid, UK) and maintained as frozen stock at -70 °C.[16]

DNA extraction accessed by the use of proteinase K, sodium dodecyl sulfate and cetyltrimethylammonium bromide.[17] For this aim, all the samples were defreeze at 37 °C in a

water bath, and 50 µl were added to Luria-Bertani broth medium and incubated at 37 °C for overnight. Finally, 300 µl of bacterial growth medium was used for DNA extraction.

DNA extracts of the identified *E. coli* strains subjected to MPCR with primer sets previously described (Table 1).[18] The target genes for identifying EAEC were pCVD432 and/or aspU and/or aggR. The positive strain that has been used for setting up of MPCR and as a positive control was EAEC strain 432, positive for pCVD432 and aspU and aggR genes.

MPCRs were performed with a 20 µl mixture containing 2 µl of template DNA, 2 µl of × 10 PCR buffer, 0.6 µl of 50 mM $MgCl_2$, 0.2 µl of 10 mM mixture of deoxy nucleoside triphosphate, 0.5 µl of 5 u/ml Taq DNA polymerase (CinnaGen Co., Iran) and 3 sets primers in concentration of 25 µM including 0.2 µl aggR, pCVD432 and aspU.[18]

The reaction mixtures were run in a thermal cycler (Eppendorf, Germany) with the following cycling profile: Denaturation at 94 °C for 7 minutes, followed by 35 cycles of denaturation at 94 °C for 50 second, annealing at 65 °C for 55 seconds and extension at 72 °C for 1 minutes, and a final extension cycle at 72 °C for 7 minutes. A negative control lacking the DNA template was included in each experiment to exclude the possibility of reagent contamination. A molecular marker (50 bp DNA ladder) was run concurrently. Amplified products were evaluated by 2% of agarose gel electrophoresis in × 0.7 Tris-borate-EDTA buffer after staining with ethidium bromide and visualized by ultraviolet light transilluminator (UVP-USA). Figure 1 demonstrates the electrophoresis result for a positive strain with patient sample.

Analyses were performed by SPSS software (version 16, SPSS Inc., Chicago, IL, USA). Categorical variables were compared by the chi-square test or Fisher's exact test. The significance level was defined as $P < 0.05$.

Results

In a 9 months period, 303 diarrheal samples from children with gastroenteritis were collected. 123 (40.6%) samples were belonging to girls. Of the total 303 children that were enrolled in this study, 247 (81.5%) were under 5 years of age.

Detection of E. coli strains

Among diarrheal specimens, 194 (64.02%) were identified as *E. coli* according to standard microbiological methods. 127 (65.5%) of them belong to boys and 67 (34.5%) were from girls.

Detection of EAEC

According to the MPCR assays, 55 (28.4%) isolates of *E. coli* were detected to have virulence genes and were considered as EAEC. Of the 55 identified EAEC strains 27 (49.1%) samples were found to harbor only aspU gene, 4 (7.3%) just pCVD432 gene and 24 (43.6%) samples were detected to have both genes (aspU and pCVD432) simultaneously. There was no sample harboring aggR gene. Regarding this results the prevalence of EAEC strains among all diarrheal samples were 18.2% (55/303). Detection rates of EAEC among girls and boys were 32.7% (18/55) and 67.3% (37/55), respectively. Based on these results the prevalence of EAEC among girls and boys were 14.6% (18/123) and 20.5% (37/180), respectively. Moreover, the prevalence of EAEC according to the age group was 17% for 0-5 years (42 of 247) and 23.2% for 5-10 years (13 of 56). However, there was no significant association between prevalence of EAEC and the age groups and also the gender of the patients ($P > 0.05$).

Table 1. PCR (polymerase chain reaction) primers used in this study

Designation	Sequence (5'→3')	Target gene	Amplicon size (bp)
Eaggfp	AGACTCTGGCGAAAGACTGTATC	CVD432	194
Eaggbp	ATGGCTGTCTGTAATAGATGAGAAC		
aggRks1	GTATACACAAAAGAAGGAAGC	aggR	254
aggRkas2	ACAGAATCGTCAGCATCAGC		
aspu-3	GCCTTTGCGGGTGGTAGCGG	aspU	282
aspu-2	AACCCATTCGGTTAGAGCAC		

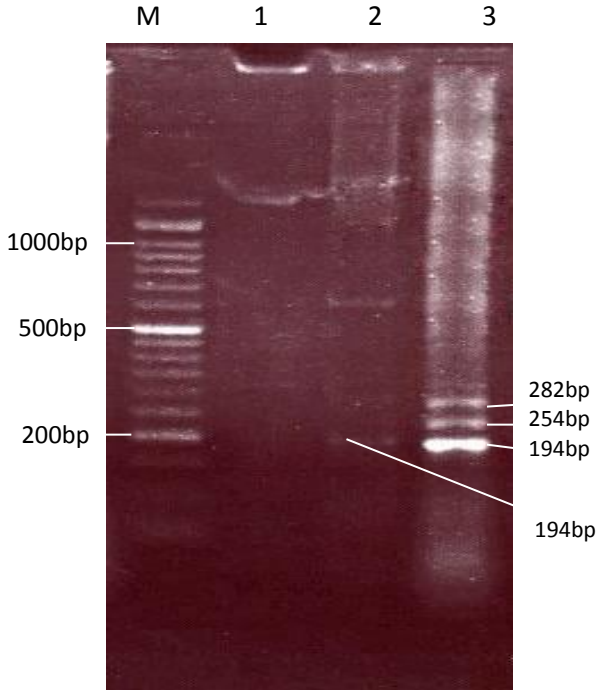

Figure 1. Electrophoresis result of positive strain and patient sample
M: Marker (50bp); 1: Negative control; 2: Patient sample (positive for pCVD432); 3: Positive strain (harboring pCVD432 and aggR and aspU genes)

Discussion

EAEC is a diarrheal pathogen of emerging importance and has been considered as one of the bacterial causative agent of acute and persistent diarrhea in children in different studies,[4,19,20] but it is not well documented in Iran, in particular, northwestern region. Routinely, the laboratories cannot detect this diarrheagenic pathotype from their counterparts that reside as normal flora. For this matter and to fill the gap of information about the frequency of this organism in the northwest of Iran, we used MPCR to detect and determine the prevalence of EAEC strains according to their virulence traits.

According to our results, the total prevalence of EAEC strains was 18.2%. It is consistent with the result of Pourakbari et al.[21] study in Tehran, Iran, that has reported 20.0% for the prevalence of these strains among children with diarrhea. The finding of our study is not in agreed with some studies conducted in Iran and other countries.[12,22-24] In a study carried out in Tehran Jafari et al.[22] reported 8.2% of prevalence rate for these diarrheagenic strains among studied cases. A similar study in Kenya has reported 8.9% of prevalence rate for these strains among children with diarrhea.[24] Likely Switzerland study showed 10.2% of prevalence rate.[23]

The common denominator in all of these described studies was the use of one virulence gene (pCVD432) as an alone marker for detection EAEC strains. Probably, because of the proved genome heterogeneity of EAEC strains, using one virulence trait for identification has decreased the rate of isolation.

Surprisingly despite using six different genes for identification EAEC strains in Hamadan, Iran, Aslani et al.[12] reported 10.7% of prevalence rate for this group. It seems limiting of EAEC strains to pCVD432 positive isolates in the first step of identification and then processing the presence of other virulence genes in this isolates could be an explanation for their low rate report.

It is clear that using pCVD432 gene alone as a marker for detection of EAEC strains in the present study can decline the prevalence rate to 9.2%.

However in a study in Egypt among children with acute diarrhea the prevalence of these diarrheagenic strains has been reported 30.7% with using pCVD432 as identification marker.[25] Time of samples collection (summer) and children's of low-income families, could be the reasons for this increased prevalence.

The prevalence of EAEC strains in present study among girls and boys were 14.6% and 20.5% and in two age groups (< 5 and 5-10 years) were 17% and 23.2% respectively.

We didn't find significant differences between the prevalence of EAEC strains and two different age group and gender ($P > 0.05$).

Not agree with our result Switzerland study showed significant association between prevalence of EAEC and children aged < 5 years, but not in older ones.[23]

Other studies have pointed to significant differences in different age groups. For example in a study conducted in Venezuela[26] difference in the prevalence of EAEC strains was significant in children with diarrhea

under 2 months age, not in older patients.

Unlike, results of a study in Nigeria[27] revealed significant differences between the prevalence of EAEC and patients older than 6 months.

Different and discordant results from different studied regions can be a consequence of strain-to-strain heterogeneity of EAEC strains in these geographical areas. However, the present study showed that there were no significant differences between isolation of EAEC strains and studied age groups or gender in this region.

A considerable interest in this study was that no sample harbor aggR gene. Regarding the suggestion for classifying EAEC to "typical" and "atypical" strains based on harboring or lacking aggR regulon respectively,[28] indicate that the isolated strains in our study are atypical EAECs. The lack of aggR in the all of EAEC-positive samples in our study showed that this gene is not a good marker to detection of EAEC strains in this region. It is consistent with report from Brazil[11] and inconsistent with some other studies.[29,30]

The presence of mutated plasmid in identified EAEC strains in our study and/or the highly heterogeneity nature of EAEC genome could be the probable reasons for explanation our finding.

The prevalence rate for pCVD432 and aspU genes in this study were 9.2% (28/303) and 16.8% (51/303) respectively. Although identification of EAEC using complementary primers for a pCVD432 gene with PCR assay has remained a more common target in different studies;[23,29,31,32] however, there are reports of detected EAEC strains associated with diarrhea lacking the pCVD432 gene.[2,27] A well-known example is an outbreak of EAEC in Japan where all isolates were negative for pCVD432 gene by PCR.[33]

Our finding showed that for identification EAEC strains based on detection presence of one gene, using aspU as target gene will give more actual results in this region.

The result of present study coincide with other similar studies indicate that using multiple virulence genes for identification of these diarrheagenic strains simultaneously is a necessity for reaching reliable results. For example in a study conducted in South India Rajendran et al.[34] reported 0.8% of prevalence for EAEC based on one gene detection, but using three different genes in a separate MPCR raised the prevalence rate up to 14.7% among studied samples. In other study from Africa Presterl et al.[20] reported 12.7% of prevalence rate for EAEC strains among children with diarrhea using pCVD432 as target gene for identification, but with raising number of identifiable target genes to five, their prevalence rate reports raised to 38%.

Conclusion

Our study revealed the high prevalence of EAEC in children with diarrhea in northwest of Iran emphasizing the fact that about 30% of *E. coli* isolates that are being report as normal flora in stool culture results, can harbor virulence genes for EAEC strains. Also using multiple known virulence genes simultaneously for detection of these strains is necessary to gain reliable results. To the best of our knowledge, this is the first report about the detection of EAEC strains among children population in northwest Iran.

Conflict of Interests

Authors have no conflict of interest.

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