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Original Article

The extended spectrum β-lactamases (ESBL) and virulence genes of intestinal enteroaggregative Escherichia coli (EAEC) in healthy elderly individuals

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Abstract: Aim: to analyze the detection rate of intestinal enteroaggregative Escherichia coli (EAEC) in healthy elderly (≥60 years) individuals in the Hangzhou area of China, and to investigate the extended spectrum β-lactamases and virulence genes of EAEC. Methods: Stool specimens provided by healthy elderly individuals were cultured on blood agar, SS, and MAC plates. The bacterial strains were identified using Vitek-2 Compact automatic microorganism identification system and mass spectrometry. The resistance phenotypes of the bacteria were determined using the double-disk synergy method. The resistance genes and the EAEC virulence gene, astA and aggR, were amplified by PCR and compared to the sequences available in Gen Bank. Results: Among the 1050 healthy volunteers, the majority of bacteria were E. coli, accounting for 960 strains, with an ESBL-positive rate of 36.3% (348/960). The EAEC detection rate was 10% (96/960); among them, 84 strains were astA, the detection rate of which was 8.75%; 12 strains were aggR, the detection rate of which was 1.25%. The ESBL-positive rate of EAEC strains were 56.25% (54/96), all of which carried the CTX-M type, with the CTX-M-14 predominating at 66.7% (36/54). Conclusions: The ESBL-positive rate of intestinal E. coli in healthy elderly individuals in the Hangzhou area of China was higher than the rate detected in other regions of china; and there was a high rate of antibiotic resistance among the intestinal EAEC in healthy elderly individuals. The results of this study suggest that EAEC is not only a pathogenic bacteria detected in diarrhea patients, but can also be present in healthy individuals, and high-resistance clinical strains have spread to the healthy population in the Hangzhou area. So vigilance is critical.

Keywords: Healthy elderly, Enterobacteriaceae, extended spectrum β-lactamases, enteroaggregative Escherichia coli

Introduction

With the increase in the elderly population in China comes an increased emphasis on the importance of health in this aging society, especially the gastrointestinal problems caused by disorders of the intestinal flora in the elderly. Escherichia coli (E. coli), which is a predominant strain in the intestinal microflora, begins its lifelong colonization of the gut a few hours after birth, providing some nutrients to the host by the synthesis of metabolites. At the same time, E. coli is an important human opportunistic pathogen, which invades the intestinal tissue or extra-intestinal organs when the host immunity is compromised, causing symptoms such as diarrhea and urinary tract inflammation. Diarrheogenic E. coli (DEC) are the main pathogenic bacteria responsible for diarrhea in third world countries. DEC can be divided into different groups according to serotype, virulence and clinical symptoms [1]. Of these, the enteroaggregative E. coli (EAEC) has attracted increasing attention in recent years due to the increased detection rate of "turista" or "traveler's" diarrhea. Furthermore, EAEC strains that were the main pathogens associated with cases of diarrhea in some areas were found exhibit high resistance to conventional antibiotics. It has been shown that the pathogenic mechanism of EAEC has an important association with adhesion and adhesion fac-

tors. EAEC gather in a characteristic "stacked-brick" arrangement on the surface of infected cells. This process is mediated by the plasmid encoded "bundle-forming pilus" and aggregation of adhesive fimbriae (AAF/I and AAF/II) regulated by the aggR gene. EAEC produces the enteroaggregative heat stable toxin (EAST-I), which is encoded by the astA gene [2].

At present, there numerous relevant reports of EAEC strains worldwide, but mostly confined to patients with diarrhea. EAEC are rarely reported in healthy people; however, due to the increased risk of gastrointestinal problems in the elderly, we focus here on investigation of EAEC in stool samples obtained from healthy patients aged ≥60 to elucidate the potential role of these strains in this population. Following conventional bacterial culture, we analyzed the virulence genes and extended spectrum beta lactamase genes to determine the distribution of intestinal EAEC in healthy elderly individuals in the Hangzhou area of China.

Materials and methods

Subject enrollment

We selected 1050 elderly (aged ≥60 years) individuals confirmed to be in good health by physical examination from March 1, 2015 to March 30, 2015, at the Healthcare Center of Zhejiang Province Traditional Chinese Medical Hospital (China). The inclusion criteria were: (1) no digestive system diseases, such as serious liver and kidney dysfunction, pancreatitis, colitis, peptic ulcer, hemorrhage, tumor, and transplantation recipients; (2) no diarrhea or use of antibiotics for the previous nearly 2 weeks; (3) hypertension, coronary heart disease and other chronic disease patients if in the stable phase of the disease. All specimens were obtained under informed consent with approval by the Ethics Committee of our hospital (Identification No. HMU (Ethics) 20121103).

Instruments and reagents

Agar (blood, SS, MH, and MCK) were purchased from BioMerieux; aztreonam (ATM), ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), and amoxicillin/clavulanic acid (AMC) were provided by OXOID; MALDI-TOF MS identification were performed by Bruker (Germany); MALDI-TOF MS reagents such as three fluorine acetic acid (TFA), alpha hydroxy phenyl cyanide-4-acrylic acid (HCCA), chromatography, formic acid, ethanol and acetonitrile were purchased from Sigma (USA); T-personal PCR amplification was performed by Biometra Germany; genomic DNA extraction kits were purchased from Axygen (USA); PCR reaction kits and DNA standards were purchased from Takara (Japan).

Isolation and identification of bacterial strains

Fresh stool samples (within 30 min of sampling) were plated onto blood, SS and MCK agar and incubated at 35°C for 24 h. Enterobacteriaceae were identified using the Vitek-2 Compact automatic microorganism identification system and MALDI-TOF mass spectrometry according to the manufacturers' instructions.

Detection of ESBL phenotype

The ESBL phenotype of all Escherichia coli by the double-disk synergy method [3]. Briefly, the AMC disk was placed in the center of the MH agar plate and disks infused with ATM, CRO, CTX, CAZ were arranged around the central disk at 25 mm intervals and at a distance of 15 mm from the edge. ESBL-producing strains were identified on the basis of the generation of a zone of growth inhibition between the AMC disk and the other disks.

Detection of EAEC virulence gene

Two EAEC virulence genes, astA and aggR, of the 960 strains of non-repetitive E. coli isolated were detected by PCR [4]. The reaction system (total volume, 25 μL) comprised 10× PCR buffer (2.5 μL), 2.5 mM dNTPs (3 μL), 5 U/μL I Taq DNA polymerase (0.5 μL), dH_2O (11 μL), primers (0.1-0.4 μL), and DNA template (2 μL). The PCR amplification conditions were: predegeneration for 5 min at 94°C, denaturation for 30 s at 94°C, renaturation for 30 s at 63°C, extension for 1.5 min at 72°C (30 cycles) with a final extension for 5 min at 72°C; samples were then maintained at 4°C. The PCR products separated by 1% agarose gel electrophoresis and visualized using a UV imaging system. EAEC strains were identified by comparison with a standard EAEC strain [4]; Strains expression any one or both of the virulence genes were deemed to be positive, and confirmed by the Hep-2 cell adhesion test.

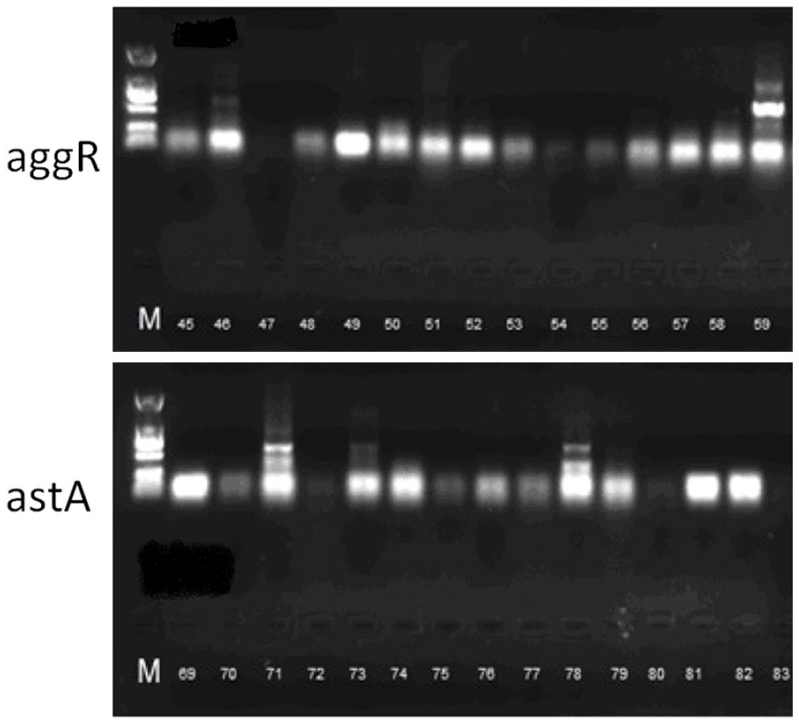

Figure 1. Agarose gel electrophoresis separation of the aggR and astA PCR product.

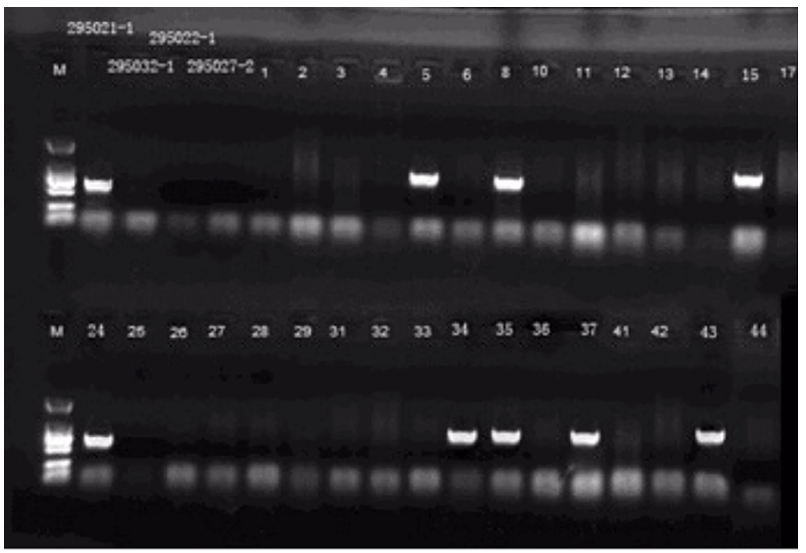

Figure 2. Agarose gel electrophoresis separation of the CTX-M-1 gene PCR products.

ESBL gene amplification

We determined the ESBL genotype of EAEC strains exhibiting an ESBL-positive phenotype using plasmid DNA as a template for PCR amplification of blaTEM, blaSHV, blaCTX-M-1, blaCTX-M-2, blaCTX-M-8, blaCTX-M-9 with previously described primers [5]. The PCR reaction system (total volume, 50 μL) was as follows: 10× PCR buffer (5 μL), Mg^{2+} (3 μL), dNTP (4 μL), Taq DNA polymerase (0.25 μL), DNA template (3 μl), primers (1 μL). The PCR amplification conditions were: 5 min at 94°C, 1 min at 94°C, 1 min at 46°C, 2 min at 72°C (30 cycles), with a final extension for 7 min at 72°C. The PCR products separated by 1% agarose gel electrophoresis and visualized using a UV imaging system.

Sequencing of ESBL and EAEC virulence genes

PCR amplification products were sequenced by Shanghai Biological Engineering (China) and the results were analyzed by comparison with the sequences available in the GenBank database.

Statistical analysis

All data were analyzed using WHONET 5.6 and SPSS 13.0 software. The t-test was used to compare the detection rate of EAEC between the ESBL-producing E. coli and the non-ESBL-producing E. coli. $P< 0.05$ was considered to be statistically significant.

Results

Detection of Escherichia coli and the ESBL phenotype

We isolated 1452 strains of Enterobacteriaceae, including 960 strains of E. coli, from stool samples of 1050 healthy subjects; the detection rate was 91.4% (960/1050). Among these,

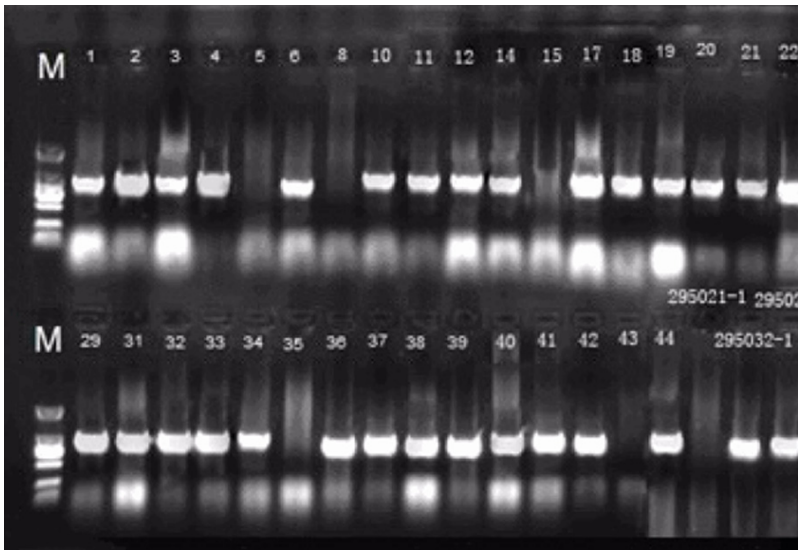

Figure 3. Agarose gel electrophoresis separation of the CTX-M-9 gene PCR products.

Table 1. EAEC virulence genes and ESBL status of 960 strains of Escherichia coli

	ESBL (+) n = 348	ESBL (-) n = 612	Total	Percentage (n = 960)
AggR	0	12	12	1.25%
AstA	54	30	84	8.75%
EAEC (astA + aggR)	54	42	96	10%
Percentage	15.51% (54/348)*	6.86% (42/612)*		

*P<0.05: the EAEC detection rate of the ESBL-producing E. coli group VS the non-ESBL-producing E. coli group.

36.3% (348/960) were identified as ESBL-positive strains of E. coli.

Amplification of ESBL and EAEC virulence genes

The EAEC virulence genes, astA (102 bp) and aggR (400 bp), were amplified by PCR and visualized following 1% agarose gel electrophoresis (**Figure 1**, respectively).

Amplification of ESBL gene

Using the extracted bacterial plasmid DNA as a template and a panel of primers designed for the amplification of ESBL genes, we amplified two fragments of 944 bp and 877 bp (**Figures 2** and **3**, respectively).

Sequencing of EAEC virulence gene

Among the E. coli strains identified, the total EAEC detection rate was 10% (96/960) and 8.75% (84/960) were astA-positive. Of these, 54 strains carried both the ESBL and astA genes, while the other 30 strains carried the astA gene alone. The aggR virulence gene was detected in 1.25% (12/960) of the E. coli strains identified, although these were not ESBL-producing strains. The detection rate of ESBL among the EAEC strains was 56.25% (54/96). The detection rate of EAEC among the ESBL-producing E. coli was significantly higher than that among the non-ESBL-producing E. coli (15.51% (54/348) vs. 6.86% (42/612); P<0.05). The astA and aggR genes were not identified in the same strain. Two E. coli strains were isolated from the same stool sample of a single healthy individual, one strain carried both the ESBL and astA genes, while the other strain carried only the aggR gene (**Table 1**).

Sequencing of ESBL genotype

All 54 ESBL-producing EAEC strains were CTX-M type, with CTX-M-14 accounting for 66.7% (36/54) and one strain carrying both CTX-M-14 and CTX-M-64 simultaneously (**Table 2**).

Discussion

Since the first ESBL strain was isolated in Germany in 1983, these antibiotic resistant strains have become an important issue in hospital infections. Recently, detection rate of ESBL strains has increased among community-acquired infections and is spreading to the healthy population. According to a study in Spain, the rate of ESBL-positive strains detected in outpatients increased from 0.7% in 1991 to 5.5% in 2003, and ESBL-producing E. coli were detected in the feces of 3.7% of healthy individuals [6]. According to a study of anal swab samples obtained from the elderly popu-

Table 2. ESBL genotype distribution of EAEC strains

Strain	CTX-M				Total
	Type -64	Type -14	Type -24	Type -27	
AggR (+)	0	0	0	0	0
AstA (+)	7	36	6	6	55
Total	7	36	6	6	55

lation in Shenyang, China, ESBL was detected in 7% of E. coli [7]. In this study, we showed that the ESBL-positive rate of intestinal E. coli in healthy elderly individuals in the Hangzhou area of China was 36.3%, which is significantly higher than the rate detected in other regions, suggesting that high-resistance clinical strains have spread to the healthy population in the Hangzhou area. This may arise due to misconceptions among the elderly in the community regarding the appropriate use of antibiotics and the relaxation of purchasing controls implemented by many large chain pharmacies. This abuse of antibiotics in the community environment has become a very serious issue that is responsible for the outgrowth of ESBL strains under antibiotic selective pressure. To prevent the increasing prevalence in antibiotic resistance, it is vital that antibiotic use is strictly controlled.

Studies have shown that EAEC strains are an important pathogen responsible for human diarrhea in both developed and developing countries. In the southern countryside in India, EAEC strains were found to be predominant among all intestinal pathogens, with a detection rate of 5.3% (64/1200) [8]. In Barcelona (Spain) in 2001, 50 (9.7%) EAEC strains were isolated from 517 patients with "traveler's diarrhea" who had recently visited developing countries [9]. In a survey of table condiments in Mexico, researchers found EAEC was found in 44% of spices [10]. Increasingly, reports show that children are highly susceptible to diarrhea caused by EAEC. Researchers in Iran found that 10.7% (15/140) of cases of diarrhea in children were caused by EAEC infection [11]. In a continuous analysis of intestinal infection in Beijing (China) reported by Qu M et al., pathogen monitoring showed that EAEC represented 15.3% [12]. However, the rate of detection of intestinal EAEC among healthy elderly individuals is rarely reported worldwide. In the present study, the two EAEC virulence genes were detected, the results showed that the rate of EAEC strains were detected at a rate of 10% based on the detection of two EAEC virulence genes; this is consistent with the rates reported in the literature. Among the EAEC identified in this study, 56.25% of strains were ESBL-positive, suggesting a high rate of antibiotic resistance among the intestinal EAEC present in healthy elderly individuals. It has been suggested there is an inverse correlation between virulence and antibiotic resistance [13], with some studies showing that the emergence of antibiotic resistance is associated with loss of virulence factors in certain E. coli. However, contradictory evidence suggests that bacterial uptake of plasmids containing resistance genes and horizontal transfer of virulence factors occur simultaneously. Contrary to this view, in the present study, the detection rate of EAEC among ESBL-producing E. coli was significantly higher than that among the non-ESBL-producing E. coli; thus, this observation is worthy of further investigation.

CTX-M is a type of ESBL that had attracted great attention in the past ten years because of its high capacity to hydrolyze cefotaxime. A number of studies in Spain have suggested that more than 60% of ESBL strains isolated from community sources are CTX-M [14]. A study conducted in Dalian, China, showed that 19 strains of E. coli producing ESBL were also CTX-M[7]. In accordance with these findings, the ESBL of EAEC strains isolated in the current study were all CTX-M type, with CTX-M-14 accounting for 66.7% (36/54). This suggests that strains producing CTX-M type ESBL may become the dominant strains in community or hospital acquired infections because of the extensive application of three generation cephalosporins such as cefotaxime.

The results of this study demonstrate that EAEC are not only detected in diarrhea patients, but are also present in the intestinal tract in healthy elderly individuals. Furthermore, two strains of EAEC isolated from the same patient had different virulence genes and antibiotic resistance, although it remains to be determined whether the virulence and antibiotic resistance genes were located on the same or different plasmids and the related risk of horizontal gene transfer remains to be investigated.

Disclosure of conflict of interest

None.

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