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Abstract

Background: Detection of the cause of diarrhoeal diseases is important for the management of the outbreaks such diseases.

Aims: This study investigated the prevalence of Shiga toxin-producing bacteria in stool samples of patients with diarrhoea associated with outbreaks of foodborne illness in the Islamic Republic of Iran.

Methods: A total of 532 stool and rectal swab samples from 70 sporadic outbreaks during May 2014 to August 2015 were examined for infection with Shiga toxin-producing bacteria. The isolates were examined for carriage of the virulence genes stx1 and stx2 in all isolates and eae/ehxA in Escherichia coli.

Results: E. coli, Shigella spp., Citrobacter spp., Enterobacter spp., Klebsiella spp. and other enteric bacteria were detected in 77.7% (376/484), 5.0% (24/484), 3.9% (19/484), 0.4% (2/484), 3.7% (18/484) and 9.3% (45/484) of the samples respectively. Of the 196 sorbitol-negative E. coli strains, 3 (1.5%) carried the stx1 gene as did 2 of the 19 (10.5%) Citrobacter strains.

Conclusion: Shiga toxin-producing Citrobacter spp. strains should be considered as a newly emerging foodborne pathogen in outbreaks.

Keywords: Shiga toxin, Citrobacter, foodborne diseases, disease outbreaks, Islamic Republic of Iran.

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Shiga toxin-producing bacteria are the main cause of bloody or non-bloody diarrhoea. They can produce a life-threatening disease known as haemolytic uraemic syndrome. While Shigella dysenteriae serotype 1 most commonly produces this toxin, other members of the Enterobacteriaceae family, such as Shiga toxin-producing Escherichia coli and enterohaemorrhagic E. coli, as well as Citrobacter spp., Enterobacter spp., Acinetobacter spp., Aeromonas spp. and Campylobacter spp., could also carry different Shiga toxin (stx) genes and their variants (stx1 and/or stx2) (1,2). Cooperation of Shiga toxins with other virulence factors, such as aggregative adhesin and intimin (eae), could induce more severe disease in infected patients (3).

The stx genes are encoded in the genome of heterogeneous lambdoid bacteriophages and can be passed to other bacteria during horizontal gene transfer (4). A high distribution of stx genes in farm or wild animals, wastewater, and land and aquatic environments suggests possible involvement of different bacterial species carrying these genes when stx-related diseases occur during outbreaks of water- and foodborne illness (5). Prompt laboratory diagnosis of these pathogens could allow more effective outbreak responses and control measures to be instituted. We therefore investigated the prevalence of stx-encoding bacterial strains and typical virulence genes (stx1, stx2, eae and ehxA) in pathogenic bacteria isolated from diarrhoeal stool samples of patients taken during sporadic outbreaks of foodborne illness in the Islamic Republic of Iran.

**Methods**

**Patients and samples**

The Center for Communicable Diseases Control of the Iranian Ministry of Health and Medical Education provided 532 stool and rectal swab samples from 70 sporadic outbreaks of foodborne illness in the Islamic Republic of Iran.
illness from 14 provinces of the Islamic Republic of Iran during May 2014 to August 2015. All data on patient symptoms and demographic characteristics were provided through a nationally approved standardized questionnaire for outbreaks of foodborne illness.

**Culture and characterization**

Fresh stool or rectal swab samples were obtained from each patient in a sterile container and transferred to the laboratory of the Foodborne and Waterborne Diseases Research Center in Cary Blair medium at 4 °C. Rectal swab samples were immediately cultured on MacConkey and sorbitol MacConkey agar media (Merck, Germany), while stool samples were enriched in Selenite F broth. To find the common Shiga toxin-producing bacteria, all the purified lactose-fermenting and non-fermenting colonies were characterized biochemically, according to the standard identification guideline (6). Serogrouping of non-sorbitol-fermenting E. coli (O157) and Shigella (A-D) strains was done using specific antisera (Baharafshan, Islamic Republic of Iran).

**Molecular characterization**

Identification of each bacterial strain and carriage of stx1, stx2, ehxA and eae genes in E. coli and stx1, stx2 and eae in non-E. coli strains was done using specific primers as shown in Table 1. DNA was extracted from the freshly grown colonies of the bacteria by a boiling method (7). All polymerase chain reaction (PCR) amplifications were done in 25 µL volumes containing 4 µL of DNA template, 0.5 mM concentrations of deoxynucleoside triphosphates, 2.5 µL of 10X PCR buffer (GeneFanavaran, Islamic Republic of Iran), 0.75 mM MgCl2, 0.3 µM concentrations of each forward and reverse primer and 0.2 U of Taq DNA polymerase (GeneFanavaran, Islamic Republic of Iran) under the following conditions: initial denaturation at 95 °C for 5 minutes, then 35 cycles of denaturation at 94 °C for 1 minute, followed by annealing at defined temperatures as shown in Table 1 for 1 minute, and finally extension at 72 °C for 1 minute.

**Analysis**

Descriptive analysis was done to report frequency of Shiga toxigenic and non-toxigenic bacteria in outbreaks of foodborne illness in the Islamic Republic of Iran. All the analysis was done using SPSS, version 17.0.

**Ethical consideration**

Ethical approval for the study was given by the Center for Disease Control and Prevention, Ministry of Health and Medical Education, and the National Institute for Medical Research
Development, Islamic Republic of Iran.

Results

The samples were obtained from patients with symptoms of diarrhoea – at least five loose stools in 24 hours, vomiting, abdominal cramp, nausea, headache and/or fever. The patients were aged between 1 and 70 years. About one fifth (21.2%) of the patients with complete demographic data were younger than 10 years. Infection of different etiology was common in the patients at aged 6–10 years.

Of the 532 samples provided, 26 (4.9%) showed no growth for bacteria and 22 (4.1%) had positive results for intestinal viruses and parasites. These samples were excluded from the study, leaving 484 samples in which bacteria were identified.

E. coli was found in 376 samples, followed by Shigella spp. in 24 samples and Klebsiella spp. in 18 samples. The clinical finding associated with the type of infection are shown in Table 2. Blood in stools was found in a greater proportion of samples with Shigella infection (12.5%) than other bacterial infections. Vomiting and abdominal pain were found in a considerably greater proportion of infections with Shiga toxin-producing E. coli and Citrobacter strains compared with non-toxigenic ones. Infection with Klebsiella spp. was detected only in patients younger than 10 years; however infection with Shigella spp. was found in all age groups.

Infection with Shigella spp. was found in samples from eight different outbreaks of foodborne illness, mostly in the spring and summer (6/8, 75.0%). Samples with high counts of Klebsiella spp. or Enterobacter spp. were also found in samples from eight different outbreaks, mostly in the autumn and winter (5/8, 62.5%). Citrobacter infection was found in samples from 10 distinct outbreaks with no seasonal tendency. Faecal carriage of E. coli was confirmed in 77.7% of the samples (376/484), while infection with Shigella spp. (5.0%, 24/484), Enterobacter spp. (0.4%, 2/484), Citrobacter spp. (3.9%, 19/484), Klebsiella spp. (3.7%, 18/484), and other enteric bacteria (9.3%, 45/484) was found in 22.3% of these samples (≥ 105 colony forming units/g).

Serological and molecular characterization

All the Shigella strains reacted with a polyvalent antiserum, defined as Shigella Poly A, and were characterized as S. dysenteriae. Serotyping of E. coli strains also verified association of
these strains with non-O157 Shiga toxin-producing E. coli serological groups. The non-O157 Shiga toxin-producing E. coli strains showed eae negative/ehxA negative genotypes.

Infection with Shiga toxin-encoding bacteria

Analysis of sorbitol fermentation for colonies grown on sorbitol MacConkey agar plates showed infection with sorbitol-negative E. coli strains in 52.1% (196/376) of the samples. Carriage of stx1 was determined in 1.5% (3/196) of sorbitol-negative E. coli and 10.5% (2/19) of Citrobacter strains. All the Shiga toxin-producing Citrobacter and E. coli isolates belonged to two distinct outbreaks in two neighbouring cities, about 80 km apart. The Shiga-toxin Citrobacter isolates were related to the same outbreak, which was reported 3 months after an outbreak caused by Shiga toxin-producing E. coli.

Discussion

Shiga toxins 1 and 2 are related toxins produced by certain bacteria and are implicated in bloody diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome and central nervous system complications (12,13). An increased number of outbreaks caused by Shiga toxin-producing bacteria, especially in developed countries, is considered an important problems in health care systems (14). While there are several reports of diarrhoea and outbreaks caused by Shiga toxin-producing E. coli serotypes, little is known about the other Shiga toxin-producing bacteria, such as Citrobacter spp., which is sporadically isolated from patients during outbreaks of food- and waterborne illness (15). We found several outbreaks where Citrobacter spp., Enterobacter spp., E. coli and Shigella spp. were isolated from the patients as the only enteric pathogens. Citrobacter is an aerobic, Gram-negative bacillus commonly found in water, soil and food, and is part of the normal enteric flora of animals and humans. Few data are available on the overall frequency of C. freundii harbouring Shiga toxins 1 and 2 in outbreaks of foodborne illness and only sporadic cases of diarrhoea are documented compared with other enteric pathogens. In fact, the involvement of Shiga toxin 2-producing C. freundii in severe diarrhoea and haemolytic uraemic syndrome is limited to two reports (16,17). A study in China investigated the presence of stx genes in 26 strains of C. freundii that were isolated from patients with diarrhoea. Their results suggest that Shiga toxin 2 is a virulence factor that plays an important role in the pathogenesis of C. freundii (18). Analysis of our results showed carriage of the stx1 gene in 10.5% (2/19) of Citrobacter strains.

To the best of our knowledge, this is the first time that the occurrence of outbreaks of foodborne illness by stx1-encoding C. freundii strains has been recorded. Since only a small proportion of these strains carried the stx1 gene, the existence of other virulence factors in this bacterium seems possible. The other virulence factors that have been proposed for diarrhoea associated
with C. freundii include heat stable toxins, cholera-like toxin and eae. The above-mentioned study in China showed that the capacity of C. freundii for aggregative adherence and cytotoxicity could explain most of its pathogenicity (18). While the emergence of stx1-encoding C. freundii in diarrhoea in our study is significant, the clinical importance and the role of these emerging strains in human pathogenicity have not yet been addressed. The spread of Shiga toxin-producing phages by horizontal gene transfer through environmental stimuli, such as antibiotics, may explain this emergence (19).

The role of non-O157 Shiga toxin-producing E. coli in the occurrence of outbreaks of foodborne illness, as well as severe diseases such as haemolytic uraemic syndrome and haemorrhagic colitis, is well known (3). Shiga toxin-producing E. coli was identified as the responsible agent in nearly two thirds of outbreaks of foodborne illness associated with vegetables in the United States of America (20). Shiga toxin-producing E. coli has been reported to be the cause of 2–40% of cases of diarrhoea in different studies (21–24). In our study, only 1.5% (3/196) of non-O157:H7 sorbitol negative E. coli strains were positive for stx1. This frequency is lower than that reported in Shiga toxin-producing E. coli in Sweden (30.3% in non-bloody diarrhoea patients) (25). This difference could be explained by the method used for characterization of Shiga toxin-producing E. coli strains, since we analysed only sorbitol negative isolates for screening of stx genes.

In conclusion, our results show the involvement of Shiga toxin-producing Citrobacter and E. coli in the occurrence of outbreaks of foodborne illness in the Islamic Republic of Iran. These results highlight the possibility for conversion of commensal intestinal bacteria to pathogenic stx-encoding strains, which is clinically important.

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