Shiga toxin-producing bacteria as emerging enteric pathogens associated with outbreaks of foodborne illness in the Islamic Republic of Iran

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Abstract

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

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 stx_1 and stx_2 in all isolates and *eae*/*ehx*A 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 stx_1 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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Introduction

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* (stx_1 and/or stx_2) *(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 (stx_1, stx_2, *eae* and *ehx*A) 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 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 stx_1, stx_2, *ehxA* and *eae* genes in *E. coli* and stx_1, stx_2 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 $MgCl_2$, 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

Table1 Primer sequences used in the study

Gene	Primer sequences 5′–3′	Length of product (bp)	Annealing temperature (°C)	Reference
eae	F: TCAATGCAGTTCCGTTATCAGTT	482	54	8
	R: GTAAAGTCCGTTACCCCAACCTG			
stx_1	F: GAAGAGTCCGTGGGATTACG	130	50	9
	R: AGCGATGCAGCTATTAATA			
stx_2	F: GGATGCATCTCTGGTCATTG	478	50	10
	R: CTTCGGTATCCTATTCCCGG			
ehxA	F: AGCTGCAAGTGCGGGTCTG	569	55	11
	R: ACGGGTTATGCCTGCAAGTTCAC			

bp: base pairs; F: forward; R: reverse.

Table 2 Clinical symptoms of patients and microscopy findings according to the bacterial species isolated from patient samples during outbreaks of foodborne illness in the Islamic Republic of Iran

Bacteria[a]	Clinical and microscopy findings No. (%)[b]					
	Vomiting	Nausea	Fever	Abdominal pain	Headache	Blood in stool
Escherichia coli (*n* = 376)	71/129 (55.0)	91/126 (72.2)	52/115 (45.2)	10/115 (8.7)	38/117 (32.5)	10/376 (2.7)
Shigella spp. (*n* = 24)	4/9 (44.4)	7/10 (70.0)	10/10 (100.0)	3/11 (27.3)	4/11 (36.4)	3/24 (12.5)
Klebsiella spp. (*n* = 18)	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)	0/18 (0)
Enterobacter spp. (*n* = 2)	NR	NR	NR	NR	NR	0/2 (0)
Citrobacter (non-toxigenic) (*n* = 17)	7/12 (58.3)	7/12 (58.3)	1/1 (100.0)	0/12 (0)	8/12 (66.6)	0/17 (0)
Shiga toxin-producing *E. coli* (*n* = 3)	2/3 (66.6)	2/3 (66.6)	1/3 (33.3)	2/3 (66.7)	0/3 (0)	0/3 (0)
Shiga toxin-producing *Citrobacter* (*n* = 2)	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)	2/2 (100.0)	0/2 (0)

NR: not reported.

[a]*Other enteric bacteria were found in 45 samples. Other enteric bacteria were found as a single infection or in coexistence with some of the bacteria shown in Table 2.*

[b]*The difference in denominators from the total number of bacteria isolated (n) is because of missing information on symptoms in the questionnaires.*

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 (≥ 10^5 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 stx_1 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 stx_1 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 stx_1-encoding *C. freundii* strains has been recorded. Since only a small proportion of these strains carried the stx_1 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 stx_1-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 *stx*1. 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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Competing interests: None declared.

Les bactéries productrices de Shigatoxines en tant que nouveaux agents pathogènes entériques associés aux flambées épidémiques de maladies d'origine alimentaire en République islamique d'Iran

Résumé

Contexte : La détection de la cause des maladies diarrhéiques est importante pour la gestion des flambées épidémiques de ce type de maladies.

Objectifs : La présente étude examinait la prévalence des bactéries productrices de Shigatoxines dans des échantillons de selles de patients souffrant de diarrhées associées à des flambées épidémiques de maladies d'origine alimentaire en République islamique d'Iran.

Méthodes : Au total, 532 échantillons de selles et d'écouvillons rectaux prélevés au cours de 70 flambées sporadiques survenues entre mai 2014 et août 2015 ont été examinés pour détecter une infection par des bactéries productrices de Shigatoxines. Les isolats ont été examinés à la recherche du portage des gènes de virulence stx_1 et stx_2 dans tous les isolats et *eae/ehx* A chez *Escherichia coli*.

Résultats : *E. coli*, *Shigella spp.*, *Citrobacter spp.*, *Enterobacter spp.*, *Klebsiella spp.* et d'autres entérobactéries ont été détectées dans 77,7 % (376/484), 5,0 % (24/484), 3,9 % (19/484), 0,4 % (2/484), 3,7 % (18/484) et 9,3 % (45/484) des échantillons, respectivement. Sur les 196 souches d'*E. coli* négatives au sorbitol, trois (1,5 %) étaient porteuses du gène stx_1, de même que deux (10,5 %) des 19 souches de *Citrobacter*.

Conclusion : Les souches de *Citrobacter spp.* productrices de Shigatoxines doivent être considérées comme un nouvel agent pathogène alimentaire lors de flambées épidémiques.

الجراثيم التي تفرز سُم الشيجا بوصفها أحد مُسببات الأمراض المعوية المُستجدَّة التي تقترن بظهور فاشيات للأمراض المنقولة بالأغذية في جمهورية إيران الإسلامية

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الخلاصة

الخلفية: من الضروري اكتشاف سبب أمراض الإسهال من أجل إدارة فاشيات هذه الأمراض حال حدوثها.

الأهداف: هدفت هذه الدراسة إلى تحري مدى انتشار الجراثيم التي تفرز سُم الشيجا في عينات البراز المأخوذة من المرضى المصابين بالإسهال المقترن بفاشيات الأمراض المنقولة بالأغذية في جمهورية إيران الإسلامية.

طرق البحث: خلال الفترة من مايو/ أيار 2014 إلى أغسطس/ آب 2015، بلغ مجموع عينات البراز والمسحات من المستقيم المأخوذة من 70 فاشيةً متفرقة، والتي فُحصت لمعرفة إذا كانت مصابة بالجراثيم التي تفرز سُم الشيجا، 532 عينةً. وفُحصت المعزولات للتعرف على نقل سُم الشيجا للجينات المسؤولة عن الشدة الفيروسية، وعلى سُم الشيجا الموجود في جميع المعزولات وسلالات eae /ehxA في الإشريكية القولونية.

النتائج: اكتُشف في العينات وجود: الإشريكية القولونية، والشيجيلا بأنواعها، والسيتروباكتر بأنواعها، والانتروباكتر بأنواعها، والكلبسيلة بأنواعها، وغيرها من الجراثيم المعوية بنسبة 77.7٪ (376/ 484)، و5.0٪ (24/ 484)، و3.9٪ (19/ 484)، و0.4٪ (2/ 484)، و3.7٪ (18/ 484)، و9.3٪ (45/ 484)، على التوالي. ومن بين سلالات الإشريكية القولونية التي جاءت نتيجة إصابتها بالسوربيتول سلبية وعددها 196 سلالة، كانت 3 منها (1.5٪) تحمل جين سُم الشيجا، كما هو الحال بالنسبة لاثنتين من سلالات السيتروباكتر البالغ عددها 19 سلالة (10.5٪).

الاستنتاجات: يجب أن تُعتبر سلالات السيتروباكتر بأنواعها التي تفرز سُم الشيجا من المسببات المُستجدة للأمراض المنقولة بالأغذية في الفاشيات.

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