Escherichia coli, *Shigella* and *Salmonella* species in acute diarrhoea in Hamedan, Islamic Republic of Iran

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وجود أنواع الإشريكية القولونية، والسلمونيلة، والشيغيلة في حالات الإسهال الحادة في هَمِدان بجمهورية إيران الإسلامية

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الخلاصة: درس الباحثون درجة تكرار ظهور أنواع الإشريكية القولونية، والشيغيلة، والسلمونيلة في عيِّنات براز المرضى المصابين بالإسهال، ممَّن يرتادون المراكز الصحية في مقاطعة هَمِدان بجمهورية إيران الإسلامية. وقد تم استفراد ذراري الشيغيلات في 17 من أصل 144 عينة (11.8٪): 10 منها شيغيلة فلكسنرية، و3 شيغيلة سونية، و2 شيغيلة بويدية، وذريَّتان لم يحدَّد نمطهما. ولم يتم استفراد أي من ذراري السلمونيلات. وعندما استُخدمت طريقة التشخيص الجزيئي، تم اكتشاف وجود الإشريكية القولونية المسبِّبة للإسهال في 37 حالة (25.7٪)، معظمها مولِّدٌ للذيفان المعوي ETEC (22 حالة) ومنها (15 حالة) من ذراري الإشريكية القولونية المنتجة لذيفان شيغا. كما وجدت عدوى مشتركة في 14 حالة (9.7٪).

ABSTRACT This study investigated the frequency of *Escherichia coli*, *Shigella* and *Salmonella* species in stool specimens from patients with diarrhoea presenting to health centres in Hamedan province, Islamic Republic of Iran. From 144 samples, *Shigella* strains were isolated in 17 cases (11.8%): 10 *Sh. flexneri*, 3 *Sh. sonnei*, 2 *Sh. boydii* and 2 untyped strains. No *Salmonella* strains were isolated. Using molecular diagnostic methods, diarrheogenic *E. coli* were detected in 37 cases (25.7%), the majority were enterotoxigenic (ETEC) (22 cases) and Shiga toxin-producing (STEC) strains (15 cases). In 14 cases (9.7%) there was co-infection.

La responsabilité d'*Escherichia coli*, des *Shigella* et des *Salmonella* dans la diarrhée aiguë dans la province d'Hamadan en République islamique d'Iran

RÉSUMÉ Cette étude a évalué la fréquence d'*Escherichia coli*, des *Shigella* et des *Salmonella* dans des échantillons de selles de patients diarrhéiques consultant dans les centres de santé dans la province d'Hamadan en République islamique d'Iran. Sur 144 échantillons, des souches de *Shigella* ont été isolées dans 17 cas (11,8 %), à savoir 10 *Sh. flexneri*, 3 *Sh. sonnei*, 2 *Sh. boydii* et 2 souches de sérogroupe inconnu. Aucune souche de *Salmonella* n'a été isolée. Les techniques de diagnostic moléculaire ont permis de détecter des *E. coli* diarrhéigènes dans 37 cas (25,7 %), dont la majorité étaient des souches entérotoxinogènes (ETEC) (22 cas) et des souches productrices de Shiga-toxines (STEC) (15 cas). Quatorze cas (9,7 %) présentaient une co-infection.

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Introduction

Diarrhoeal diseases are among the most common causes of death in the world [1,2] with a greater medical and economic burden in developing countries. In spite of the lack of accurate investigations in developing countries, the problem of diarrhoeal diseases in these regions is much greater than it seems [3].

Escherichia. coli, *Shigella* and *Salmonella* spp. are among the most important agents causing diarrhoea worldwide [3]. At least 5 different categories of *E. coli* are responsible for diarrhoea: enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and entero-aggregative *E. coli* (EAEC). EHEC excrete potent toxins called verotoxins or Shiga toxins and are referred to as Shiga toxin-producing *E. coli* (STEC).

The associated diseases comprise: childhood and traveller's diarrhoea (ETEC), bloody diarrhoea and haemolytic uraemic syndrome (EHEC), infantile diarrhoea (EPEC), and bacillary dysentery-like diarrhoea (EIEC). EAEC has been associated with acute and persistent diarrhoea in children and adults in industrialized and developing countries in Europe, America, Asia and Africa [4,5].

Shigella is an important source of bacterial diarrhoea in developing countries [6–8]. The most important diarrheogenic *Shigella* strains are *Sh. flexneri*, *Sh. sonnei*, *Sh. boydii* and *Sh. dysenteriae* and untyped *Shigella* strains. *Salmonella* is another etiological factor in diarrhoea and the most common source of bacterial diarrhoea in developed countries [9]. It consists of serotypes A, B, C, D and *S. typhi*.

The pattern of major diarrhoea pathogens in the Islamic Republic of Iran seems to be different from that of many developed countries. *E. coli* species are the most frequently isolated pathogens, followed by *Salmonella*, *Shigella*, and *Campylobacter* [3,7,8], while in developed countries *Campylobacter* is more prevalent [10]. Developments in molecular analysis techniques have increased our ability to identify potential enteropathogens in stool specimens, and, in recent years, several new agents have been increasingly recognized in association with diarrhoeal disease [10]. There is little data about the molecular epidemiology of these in the Islamic Republic of Iran. Thus we decided to investigate the frequency of diarrheogenic *E. coli*, *Shigella*, *Salmonella* and their strains using both traditional and molecular diagnostic methods in patients with acute diarrhoea in Hamedan province.

Methods

Area and population

The provincial capital, also called Hamedan, is located 343 km south west of Tehran at the foot of Mount Alvand (3580 m) in the Zagros range, at an altitude of 1700 m, and dominates the wide, fertile plain of the upper Qareh Su River. It has long and severe winters from September to May with heavy snow and temperate summers.

Specimens

Between June 2003 and August 2003 all patients attending the health centres of the Hamedan province health network for acute diarrhoea were enrolled in the study. For every patient a fresh stool sample was taken and transported in less than 6 hours to the central laboratory in Cary-Blair transport medium and processed within 2 hours. All the specimens were examined for *E. coli*, *Shigella* spp. and *Salmonella* spp.

Bacteriologic methods

All samples were cultured on MacConkey agar, *Shigella* agar, *Salmonella* agar, xylose lysine decarboxylase (XLD), sorbitol Mac-Conkey and bismuth sulfite MacConkey and incubated for 24 hours at 37 °C. The detection of *Shigella* and *Salmonella* spp. was conducted using traditional microbiological methods and with commercial antisera if necessary. Polymerase chain reaction (PCR) of mixed culture was used for the detection of diarrheogenic *E. coli* strains.

DNA was extracted from the primary mixed culture of faecal samples and was subjected to 6 different PCR reactions targeting STEC (*stx1* and *stx2* genes), ETEC (LT and ST toxin-producing genes), EPEC (*eae* gene), and EAEC (*pCVD432* plasmid) respectively [*10–14*] (Table 1).

For PCR, a loopful of Gram-negative bacterial growth was taken from the first streaking area of the primary culture plate and was suspended in 0.5 mL of sterile distilled water and boiled for 20 minutes. Extracts of DNA from mixed culture were subjected to PCR by 6 pair of primers tar-geting *stx1*, *stx2*, *eae*, *pCVD432* plasmid, LT and ST.

From the PCR positive primary faecal culture, distinct *E. coli*-like and other Gram-negative colonies were isolated and tested for the presence of sequences which had initially given a positive result. As many colonies as required for finding the isolate carrying these particular genes were assayed. The isolates were subsequently characterized biochemically according to standard methods. *E. coli* strains that carried *eae* and were negative for *stx* were interpreted as EPEC. The strains positive in PCR for *pCVD432* were interpreted as EAEC, and those positive for *stx* genes as STEC. *E. coli* strains positive in PCR for LT, ST or both were considered ETEC. Positive controls were *E. coli* EDL 933, *E. coli* RH 4283, *E. coli* RH 4260, *E. coli* ATCC 35401, *E. coli* 43886 and *E. coli* ATCC 35401 and *E. coli* ATCC was a negative control.

Analysis

Descriptive statistics for patients were reported and continuous variables were sum-

Table 1 **Target genes, primers, amplicor size, and references of the primers**

Target	Primer sequence (5'–3')	Amplicor size (bp)	Reference of primers
STEC/*stx1*	CAGTTAATGTGGTGGCGAAG CTGCTAATAGTTCTGCGCATC	894	(13)
STEC/*stx2*	CTTCGGTATCCTATTCCCGG GGATGCATCTCTGGTCATTG	478	(13)
EPEC/*eae*	TGCGGCACAACAGGCGGCGA CGGTCGCCGCACCAGGATTC	629	(14)
EAEC/*pCVD432*	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	(15)
ETEC/*LT*	TCTCTATATGCACACGGAGC CCATACTGATTGCCGCAAT TCTCTATGTGCATACGGAGC	321	(13)
ETEC/*ST*	TCTGTATTGTCTTTTTCACC TTAATAGCACCCGGTACAAGC	186	(16)

marized as mean and standard deviation (SD). Patients were analysed in 2 groups: ≤ 5 years and > 5 years. The chi-squared test was used to compare categorical variables between age groups and etiology of disease. The level of significance was $P < 0.05$.

Results

We collected a total of 144 samples from patients with acute diarrhoea: 3 samples could not be cultured. The male to female ratio of patients was 1.52:1. The mean age was 20.2 years (range 5 months to 90 years); 67 patients were in the age group ≤ 5 years.

Shigella strains were isolated in 17 (11.8%) cases: 10 *Sh. flexneri*, 3 *Sh. sonnei*, 2 *Sh. boydii* and 2 untyped *Shigella* strains. *Shigella* were found in 8 (11.9%) of the age group ≤ 5 years and 9 (11.7%) of the > 5 years age group.

No *Salmonella* strains were isolated.

In the molecular analysis, diarrheogenic *E. coli* were detected in 37 cases (25.7%): 2 (1.3%) EPEC, 10 (7.0%) EAEC, 22 (15.2%) ETEC and 15 (10.4%) (STEC).

In 14 patients (9.7%) there was co-infection: 9 patients (6.3%) had 2 or more types of diarrheogenic *E. coli*, 4 patients (3.0%) had 1 diarrheogenic *E. coli* strain and *Shigella* and 1 patient had 2 diarrheogenic *E. coli* strains and *Shigella*. *E. coli* were found in 18 (26.9%) patients aged ≤ 5 years and 24.7% of those aged > 5 years.

There was no relation between age and contamination with the bacterial agents. Furthermore such comparison was taken for different strains of diarrheogenic *E. coli* and similar results were seen for them. On the other hand there were no differences between these diarrheogenic *E. coli* in these 2 age groups. Table 2 shows the frequency of bacterial pathogens in the 2 age groups.

Table 2 **Frequency of bacterial pathogens in the age groups above and below 5 years**

Pathogen	Age group (years)	
	≤ 5	> 5
	No.	No.
Escherichia coli		
Enteropathogenic	1	1
Enteroaggregative	5	5
Enterotoxigenic	12	10
Shiga toxin-producing	7	8
Shigella spp.		
Sh. flexneri	6	4
Sh. sonnei	2	1
Sh. boidae	1	1
Untypeable	1	1

Discussion

Diarrhoeal diseases are a major problem in the Islamic Republic of Iran, as in other developing countries [11,12]. Clarification of the enteropathogens associated with diarrhoeal diseases in the country is an essential step towards the implementation of effective primary health care prevention activities against the diseases. The present etiological study is also meaningful in that it provides information on the prevalence of 3 important enteropathogens in the Hamedan area of the west of the Islamic Republic of Iran. The specimens analysed were taken from patients who had diarrhoea severe enough to bring them to a medical facility.

E. coli spp. played an important role in diarrhoea in all age groups. The frequency of isolation of *E. coli* spp. was 25.7% on average, which is markedly higher than that reported in other developing countries (11.0% and 16.9%) (Somalia and Thailand) [12,13] and previously in the Islamic Republic of Iran (16.2%, 6.8%) [14,15].

Almost all studies in this country have been performed on children and other age groups have been ignored. In this study all age groups were included and we did not detect any differences between the frequencies of this organism between the 2 age groups. So in our study, the frequency of *E. coli* in children (26.9%) was markedly higher than another study in the Islamic Republic of Iran from Islamshahr, south Tehran (6.8%) [*15*].

ETEC was detected in 22 patients (15.2%) and was the most common strain of diarrheogenic *E. coli* isolated, a finding that is consistent with previous studies which reported frequencies of ETEC of 22% [*16*]. This organism can be assumed to be one of the most important diarrheogenic *E. coli* strains.

The second most frequently detected diarrheogenic *E. coli* strain was STEC, detected in 15 cases (10.4%). In 2 studies the frequency of this strain was reported in the normal population in 2 provinces of the Islamic Republic of Iran (4.9% and 0.7% of people respectively) and with a greater frequency in the age group < 6 years [*17,18*]. Our data showed a higher frequency in comparison with Sweden (8%) where this strain is not a predominant agent among diarrheogenic *E. coli* [*19*]. STEC has an important role in fatal haemolytic uraemic syndrome, which is believed to be one of the most frequent causes of acute renal failure in children [*20*], and therefore more focus may be needed on the diagnosis of this organism using molecular methods such as PCR.

The EAEC strain is a new category of diarrheogenic *E. coli* and we detected this pathogen in 10 patients (7%), which is comparable with other studies conducted in Thailand and Brazil, where its frequency among children with acute diarrhoea was reported to be 12% and 11% respectively [*11,12*]. This contrasts with the low frequency of this organism in developed countries, for example in a study in western Europe its frequency was reported to be 2% in children with acute diarrhoea and 0% in individuals without diarrhoea [*21*].

We detected the EPEC strain in 2 patients (1.3%), and it was ranked fourth of the causes of diarrheogenic *E. coli*, whereas in another study in 1988 in the Islamic Republic of Iran this organism was the most frequently detected strain (31%) [*16*]. Despite differences between the methods used, this finding suggests a declining role of this organism in acute diarrhoea.

Shigella is a important pathogen in developing countries [*13,22*] and one of the most important causes of acute diarrhoea in the Islamic Republic of Iran in children and in adults [*7,8*]. The isolation frequency increases progressively with age [*23,24*]. In this study *Shigella* spp. were isolated in 17 (11.8%) of patients. We did not find any difference between children and adults (11.8% versus 11.7%). In other studies, *Shigella* spp. were associated with diarrhoea in children in a relatively older age group [*3*].

In general, *Sh. sonnei* is reported to cause the majority of cases of shigellosis in industrialized countries, and in developing countries where hygiene is poor. The incidence of other *Shigella* species such as *Sh. dysenteriae*, *Sh. boydii* and *Sh. flexneri* are relatively high [*9*]. In the present study, *Sh. flexneri* and *Sh. sonnei* accounted for 58.8% and 17.6% of all *Shigella* isolates, respectively. Considering these frequencies, the pattern of prevalence of each *Shigella* species in the Islamic Republic of Iran can be considered the same as that in a developing country. In studies in Kuwait and Nigeria *Sh. flexneri* was also the predominant isolated strain among *Shigella* spp. (46.0% and 51.6%) [*25,26*].

In our study we did not detect any cases of *Salmonella* infection, while in other studies in this country *Salmonella* was an important strain in bacterial diarrhoea (14.9%) with *S. enteritidis*, *S. typhimurium*, and *S. typhi* as the most common species [*3–15, 27*]. In a study in 1986 *Salmonella* was the most common diarrheogenic pathogen in our country [*28*] but later studies found this organism in second and third rank [*15,27*]. This finding is surprising as the reservoir of infection in animals constitutes the principal source of infection with *Salmonella* spp.

[*29*] and in view of the presence of animals in people's daily lives in the Islamic Republic of Iran, we expected a higher frequency of isolation of *Salmonella* spp.

Conclusion

We recommend that comprehensive surveys are needed in different parts of the Islamic Republic of Iran in order to identify the incidence of different diarrhoeal pathogens in the country.

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