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Diarrheagenic *Escherichia coli* and *Shigella* with High Rate of Extended-Spectrum Beta-Lactamase Production: Two Predominant Etiological Agents of Acute Diarrhea in Shiraz, Iran

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This study was conducted to find the etiology of acute diarrhea in Iranian children and determine the antimicrobial resistance patterns. The pathogenic bacteria were recovered from 110/269 (40.9%) diarrheal fecal samples with the following profiles: the most predominant pathogen was diarrheagenic *Escherichia coli* (DEC) (43.6%), comprising enteroaggregative *E. coli* (23.6%), enteropathogenic *E. coli* (10.9%), enteroinvasive *E. coli* (5.5%), and enterotoxigenic *E. coli* (3.6%); *Shigella* spp. (37.3%), *Salmonella* spp. (12.7%) and *Campylobacter jejuni* (6.4%) were ranked second and fourth in terms of prevalence, respectively. The rates of extended-spectrum beta-lactamase (ESBL) production were 66.7% and 53.7% in DEC and *Shigella*, respectively. Resistance to ampicillin (AMP) (95.1%), trimethoprim/sulfamethoxazole (SXT) (73.2%), azithromycin (ATH) (21.9%), and ciprofloxacin (CIP) (14.6%) was observed among *Shigella* isolates. Multidrug resistance phenotype was observed in 24.4% (10/41) of *Shigella* isolates, with the most common pattern of resistance to cefotaxime, ceftriaxone, ceftazidime, AMP, SXT, and ATH. This study indicates an alarming increase in the ESBL production of DEC and *Shigella* spp. and identifies them as the two most prevalent diarrhea-causing enteropathogens in the region. The results show that CIP could be an alternative to third-generation cephalosporins against these two pathogens. Therefore, it is proposed that further investigation be done in the pursuit of alternative antibiotics that are effective against the resistant cases. For instance, one study could look into the comparative clinical effectiveness of third-generation cephalosporins versus CIP, the latter not being presently the drug of choice for the treatment of acute diarrhea in children in Iran.

Keywords: acute diarrhea, enteropathogenic bacteria, diarrheagenic *E. coli*, *Shigella*, ESBL, MDR

Introduction

Acute diarrhea is a leading cause of morbidity and mortality worldwide, still remaining a major global health problem, especially among children in developing countries. It is responsible for the death of 1.5 million people every year (WHO, 2012). In the year 2011, 700,000 children under the age of 5 did not survive complications of diarrhea in 15 high-burden countries.[1] In Iran, it has been estimated that diarrhea is responsible for 18 million cases of illness annually, leading to the death of 516 children younger than 5 years of age.[2]

The etiology of acute diarrhea differs significantly in developed and developing countries.[3] In developed countries, in most cases, viruses are identified as the etiology of acute diarrhea,[4,5] while in developing countries, acute diarrhea is mostly attributed to bacterial pathogens. However, a number of different bacterial pathogens such as *Salmonella*, *Campylobacter*, *Shigella*, and diarrheagenic *Escherichia coli* (DEC) with different frequency patterns are isolated as the causes of acute diarrhea in both developed and developing countries.[3]

DEC represent one of the most common etiological causes of diarrhea in children worldwide.[6] Among the *E. coli* that cause diarrhea, there are at least six well-described groups, each corresponding to a distinct clinical syndrome with distinct epidemiological and pathologic schemes.[7] These organisms are currently classified as follows: enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli*.[6]

The control of infectious diseases has become seriously problematic due to an increase in the number of organisms that

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are no longer susceptible to antimicrobial drugs. Resistance to antibacterial drugs in enteric bacteria is frequently reported in developing countries.[8] Currently, multidrug-resistant (MDR) organisms, which had been initially found in hospitals, are now found almost with the same frequency within the community.[8] β-Lactam antibiotics are the most commonly prescribed antibiotics. The major mechanism of β-lactam resistance in enterobacteriaceae is the production of extended-spectrum beta-lactamases (ESBLs), conferring resistance to all β-lactams except cephamycin and carbapenems.[9]

An understanding of the etiology of diarrhea and patterns of antimicrobial resistance is important for both local epidemiological surveillance and the empirical treatment. Systematic surveillance systems are usually absent in developing countries. There are, however, limited data on the etiology of diarrhea in the developing world, both in terms of geography and pathogen specificity.[10–14] In Iran, for instance, there are very few reports to date on the etiology of diarrhea caused by enteric pathogens.[10,11,15] Even so, most of the existing reports focus on the prevalence of a specific pathogen as the causative agent.[16–21] Multiple studies have indicated that resistance may be increasing among enteric pathogens in Iran, as there are fewer restrictions on the use of antimicrobials among populations of both humans and livestock.[15,19,22–25] The aims of this study were to detect common bacterial pathogens associated with diarrhea in Iranian children using a combination of biochemical, microbiological, and molecular diagnostic techniques, and to further determine their antimicrobial resistance and ESBL production patterns.

Materials and Methods

Study population

Acute diarrhea is defined as at least three loose stools within 24 hours, lasting not more than 14 days.[2] Included in this study were children under the age of 18 who, complaining about diarrhea, referred to the emergency wards of three major hospitals in Shiraz (*i.e.*, Nemazee, Dastgheyb, and Hejazi Hospitals) between August 2014 and February 2015. These were asked, at the time of referral to hospital laboratories, whether they would give their consent that in addition to the tests ordered by their physicians, other tests be done on their stool samples. They were also told that they would be informed of the results of such tests once they were ready. All the patients (or their parents in the case of minor children) gave their consent to this request. A questionnaire had been prepared for every child to collect the following information: age, sex, clinical symptoms (*e.g.*, fever and abdominal pain), type of diarrhea, and the possible use of antibiotics before stool sampling.

Direct stool examinations were done on all stool samples in the laboratory of the hospital where samples were obtained. Two hundred sixty-nine stool samples, which were positive for white blood cell (WBC) in direct examination, were sent to our laboratory (Professor Alborzi Clinical Microbiology Research Center, Shiraz, Iran), where all the study's experiments were done.

Sample processing

Patients' fecal samples were transferred in the Cary-Blair transport medium within the maximum of 48 hours after sample collection and processed within 2 hours of their reception in our laboratory. All stool samples were investigated for *Salmonella*, *Shigella*, *Campylobacter*, and DEC. To isolate *Salmonella* spp. and *Shigella* spp., stool samples were treated in two different ways: they were inoculated directly on MacConkey agar, Xylose-Lysine-Deoxycholate (XLD), and Hektoen Enteric agar (HE) (Merck, Darmstadt, Germany) and then incubated at 37°C for 24 hours. They were also initially inoculated overnight on Gram Negative (GN) selective enrichment broth before being subcultured on XLD and HE agar. Concerning *Campylobacter* spp., the samples were inoculated on the selective medium for this bacterium—Skirrow's medium. The latter was made using Columbia Agar (Biolife) as the base, to which was added 7% of lysed horse blood as well as vancomycin, trimethoprim, and polymyxin B (Sigma) with final concentrations of 0.01 g, 0.005 g, and 2,500 IU/L, respectively. The plates were then incubated at 42°C for 2–3 days under microaerophilic conditions (10% CO_2, 5% O_2, and 83% N_2) created in an Anoxomat (MART®; Microbiology. B.V.).

All the colonies suspected for *Shigella* and *Salmonella* from HE and XLD agar were identified by biochemical tests; and to confirm, API 20E (Biomérieux, France) was performed. Serotypes of all the *Shigella* isolates were determined using commercially available antisera (Microgen Bioproducts, Ltd., Camberley, United Kingdom) against all *Shigella* serotypes. All the *Salmonella* isolates underwent serotyping—first with poly OMA and OMB, and subsequently with commercially available antisera (Microgen Bioproducts, Ltd.) that are against *Salmonella* serogroups A, B, C, and D, and *vi* antigen. Biochemical reactions were used to differentiate between two species and six subspecies of *Salmonella* isolates.[26]

For DEC identification, all *E. coli* colonies on MacConkey agar were preserved at −70°C, to be used at a later stage for group typing by PCR. All the colonies suspected for *Campylobacter* were examined in wet smear as well as microscopically after Gram staining. *Campylobacter* was confirmed by 23S rRNA PCR, and species were identified using primer sets as presented in Table 1.

Molecular diagnostic methods for the detection of pathogenic E. coli virulence genes

The DNAs were extracted using the polyethylene glycol (PEG)200 alkaline buffer method.[27] To identify different groups of *E. coli*, their associated specific primers were utilized. These included *bfp* and *eae*A for EPEC; *lt* and *st* for ETEC; *ipa*H and *vir*F for EIEC; *stx1*, *stx2*, and *eae*A for EHEC; and *agg* and *aap* for EAEC (Table 1). PCR was performed in the final volume of 50 µl, including 5 µl PCR buffer (Thermo Scientific; Maxima Hot Start *Taq* DNA polymerase, EP0602), 2.5 mM of $MgCl_2$ (Thermo Scientific; Maxima Hot Start *Taq* DNA polymerase, EP0602), 0.4 ng of mixed dNTP (Thermo Scientific; R0192), 15 pmol of each primer (Bioneer, South Korea), 2.5 U of *Taq* polymerase (Thermo Scientific; Maxima Hot Start *Taq* DNA polymerase, EP0602), and 2 µl of template. The solutions were then subjected to the following cycling condition: 94°C for 5 minutes, 94°C for 30 seconds, 55°C for 30 seconds (for *st* gene, the optimal annealing was at 50°C), and 72°C for 30 seconds (35 cycles), and a final extension step (72°C for 8

Table 1. List of Primers Used

Tested strains	*Locus*	*Primers*	*Amplicon size (bp)*	*Ref*
EPEC	*bfp*	F: AATGGTGCTTGCGCTTGCTGC R: GCCGCTTTATCCAACCTGGTAAG	326	[49]
	*eae*A	F: GACCCGGCACAAGCATAAGC R: CCACCTGCAGCAACAAGAGG	384	[50]
ETEC	*lt*	F: GGCGACAGATTATACCGTGC R: CGGTCTCTATATTCCCTGTT	450	[49]
	st	F: ATTTTTCTTTCTGTATTATCTTT R: CCGGTACAAGCAGGATTACA	190	[49]
EIEC	*ipa*H	F: ATGCGAGAAATTAATATGCTCAG R: GAATAGCAGAGTTTGATCTGATAAG	786	This study
	*vir*F	F: AGCTCAGGCAATGAAACTTTGAC R: TGGGCTTGATATTCCGATAAGTC	618	[51]
STEC	*stx1*	F: TTCAGCAAGTGCGCTGGCGA R: CGCTGAATCCCCCTCCATTA	212	This study
	stx2	F: GGCGCGTTTTGACCATCTTCG R: GATGATGGCAATTCAGTATAACG	518	This study
EAEC	*agg*R	F: GTATACACAAAAGAAGGAAGC R: ACAGAATCGTCAGCATCAGC	254	[51]
	aap	F: GGCATCTTGGGTATCAGCCTG R: CCCATTCGGTTAGAGCACTATATT	313	This study
Campylobacter jejuni	*hip*O	F: ACTTCTTTATTGCTTGCTGC R: GCCACAACAAGTAAAGAAGC	323	pubmlst.org/campylobacter
Campylobacter coli		F: TCAAGGCGTTTATGCTGCAC R: CCATCACTTACAAGCTTATAC	323	pubmlst.org/campylobacter
Campylobacter	23 rRNA	F: TATACCGGTAAGGAGTGCTGGAG R: ATCAATTAACCTTCGAGCACCG	650	pubmlst.org/campylobacter

EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EIEC, enteroinvasive *E. coli*; STEC, Shiga toxin-producing *E. coli*; EAEC, enteroaggregative *E. coli*; *bfp*, bundle-forming pilli; *eaeA*, intimin, effacing and attaching lesion; *lt*, heat labile toxin; *st*, heat-stable toxin; *ipa*H, invasion plasmid antigen; *vir*F, virulence invasion factor; *stx*1, shiga toxin1; *stx*2, shiga toxin 2; *agg*R, aggregative adherence fimbriae I; *aap*, antigenic antiaggregative protein; *hip*O, hippurate hydrolysis.

minutes) in a thermal cycler (Applied Biosystem, Veriti). Subsequently, 8 µl of the PCR product was subjected to gel electrophoresis (Biorad; Wide mini-sub® Cell GT) employing 1.5% Agarose (Invitrogen; 16500), stained by means of GelRed Nucleic Acid Gel Stain (Biotium; 41002), and visualized by gel documentation (UVItec; DBT-08). In each PCR run, genomic DNA from *E. coli* ATCC 35401 (*lt*+ and *st*+), *E. coli* ATCC 43887 (*stx*1−, *stx*2−, and *eae*+), *E. coli* containing pCVD432 (*agg*R+ and *aap*+), and *E. coli* O157:H7, ATCC 43894 (*stx*1+, *stx*2+, and *eae*+) was used as positive controls.

E. coli strains that were positive for *agg*R or/and *aap* genes were interpreted as being EAEC. The strains that carried *eae* gene, but were negative for *bfp*, *stx*1, and *stx*2 were interpreted as being atypical EPEC, and those positive for *stx*1 and *stx*2 as Shiga toxin-producing *E. coli*. The EIEC were the ones that were positive for *vir*F or/and *ipa*H. Finally, the *E. coli* strains positive for *lt*, *st*, or both were considered ETEC (Table 1).

Antimicrobial susceptibility testing

To determine the susceptibility of *C. jejuni* isolates to antimicrobial agents, isolates were inoculated on Muller-Hinton agar with 5% of horse blood. Discs containing ciprofloxacin (CIP, 5 µg), azithromycin (ATH, 15 µg), gentamicin (GM, 10 µg), tetracycline (TET, 30 µg), nalidixic acid (NA, 30 µg), ampicillin (AMP, 10 µg), and meropenem (MRP, 10 µg) (Rosco Neo-Sensitabs, Denmark) were placed on the inoculated plates. The plates were then incubated in a microaerophilic condition at 42°C for 24 hours. The results were then interpreted according to EUCAST 2015 breakpoints.[28]

Susceptibility tests were performed for other isolated pathogens to 10 commercially available antibiotics (Rosco Neo-Sensitabs), using the Kirby-Bauer disc diffusion method on Muller-Hinton agar according to CLSI 2015 guidelines and breakpoints.[29] The antibiotics used comprised cefotaxime (CTX, 30 µg), ceftriaxone (CTR, 30 µg), ceftazidime (CAZ, 30 µg), AMP (10 µg), amikacin (AMK, (30 µg), trimethoprim/sulfamethoxazole (SXT, 25 µg), GM (10 µg), CIP (5 µg), MRP (10 µg) and ATH (15 µg). A minimum of two independent experiments were performed to identify the resistant phenotype of the isolated pathogen against each antibiotic. Minimum inhibitory concentration (MIC) against *Shigella* and DEC isolates was determined for CTX, CTR, CAZ, CIP, and ATH, using the microbroth dilution method according to CLSI 2015.[29]

In this study, isolates are classified as MDR if they are resistant to at least three antimicrobial agent classes.[30]

ESBL production

Isolates with reduced susceptibility to CTX, CAZ, or CTR were screened to see if they are ESBL positive through the CLSI 2015 combination disc method,[29] using discs of CTX and CAZ along with those with clavulanic acid added (Rosco

Neo-Sensitabs). A zone diameter of ≥5 mm for the latter disc, which was larger than either of the agents tested alone, was taken as evidence for a positive ESBL production. Zone diameters were determined using the HiAntibiotic zone scale (Himedia). A minimum of two independent experiments were performed in the case of each isolated pathogen.

Results

A total of 269 samples from patients (with a male–female ratio of 1.21:1) with acute diarrhea were analyzed. Since we did not have the possibility of having a staff member in each emergency location, the prepared questionnaires were mostly unfilled, depriving us of patients' clinical information.

Enteropathogenic bacteria were isolated from 110 (40.9%) of the 269 samples collected from patients younger than 18 years with acute diarrhea (Table 2). From among these, 99 cases (89.9%) comprised a single infection, while in the remaining 11 (10.1%) cases, multiple bacterial pathogens were identified. A variety of different mixed infections were observed, most cases of which were a combination of *Shigella* with one or two groups of DEC. The combinations and frequencies of mixed infections are shown in Table 3.

Prevalence of enteric pathogens

The main enteropathogenic bacteria isolated in this study are shown in Table 2. The most common (48/110 or 43.6% of positive cases) was DEC, the most prevalent of which were the groups EAEC, EPEC, EIEC, and ETE comprising 23.6%, 10.9%, 5.5%, and 3.6% of DEC cases, respectively (Table 2). Enterohemorrhagic *E. coli* was not detected in this group. Of the 26 EAEC, 20 isolates were positive for both *agg*R and *aap* genes, while 5 and 1 isolates were positive, respectively, only for *aap* and *agg*R. All the 12 EPEC isolates were positive for *eae* gene, but negative for *bfp* and *stx* genes. In all the six *E. coli* isolates identified as EIEC, both *vir*F and *ipa*H were detected. Of the six isolated ETEC strains, *lt* genes were detected in three and *st* ones in two. Among ETEC isolates, only one isolate was positive for both *lt* and *st* genes. In our study, *Shigella* spp. was the second most prevalent pathogen isolated, the prevalence being 37.3% of positive cases. Out of the 41 *Shigella* isolates, 33 were identified as *S. flexeneri* (80.5%) and the rest as *S. sonnei*. *Salmonella* spp. was found in 14 samples, representing 12.7% of positive cases. Based on their utilization pattern of sugars and amino acids, all isolates were further identified as *S. enterica* subspecies I (enterica).[26] *Campylobacter* was found in 6.4% of positive samples. Based on PCR results, in which specific primer sets for different species were employed, all the seven *Campylobacter* isolates turned out to belong to the *C. jejuni* (Table 2).

Table 2. Prevalence of Different Enteropathogenic Bacteria in Children with Acute Diarrhea in Whom a Pathogen Was Identified ($N = 110$)

Enteropathogen	*Number of isolates (%)*[a]
Shigella spp.	41 (37.3)
Salmonella enterica (I)[b]	14 (12.7)
Campylobacter jejuni	7 (6.4)
Diarrheagenic *E. coli*	48 (43.6)
EAEC	26 (23.6)
*agg*R$^+$	1 (3.8)
aap$^+$	5 (19.2)
*agg*R$^+$, *aap*$^+$	20 (77)
EPEC	12 (10.9)
EIEC	6 (5.5)
ETEC	4 (3.6)
lt	2 (50)
st	1 (25)
lt, *st*	1 (25)
Total	110 (100)

[a]Percentage of children from whom the indicated pathogen was isolated or detected.
[b]Subspecies enterica.

Table 3. Mixed Infections Found in Children with Acute Diarrhea

Pathogenic bacteria	*No. of patients*
Shigella/EPEC	1
Shigella/EAEC	3
Shigella/EPEC/ETEC	1
Shigella/EAEC/EAEC/ETEC	1
Salmonella/*C. jejuni*	1
Salmonella/EAEC	1
C. jejuni/EAEC	2
EAEC/EPEC	1
Total	11

Antimicrobial susceptibility testing and ESBL production

All 110 enteric pathogens isolated from the stools of children in this study went through antimicrobial susceptibility testing for the 10 selected antibiotics, which are among the antibiotics suggested by CLSI 2015 for routine testing and reporting on enterobacteriacae.[29] Table 4 presents the rate of resistance to different antibiotics as well as ESBL production for enteropathogenic bacteria. The rate of ESBL-positive cases in DEC isolates was 66.7%, with the highest rate observed in the EAEC (84.6%) and the lowest in the EPEC (33.3%). Out of 48 DEC isolates, 31 (64.6%) were MDR. All DEC with the MDR phenotype were resistant to AMP and SXT, as well as to the third-generation cephalosporins (CTX, CTR, and CAZ). Of the 31 resistant isolates, 8 showed resistance to CIP, too. No evidence of MRP resistance was observed among diarrhea-causing *E. coli*.

Over 50% of *Shigella* isolates were resistant to third-generation cephalosporins and positive for ESBL production. These isolates showed a high level of resistance to AMP (100% in *S. flexneri* and 75% in *S. sonnei*) and SXT (66.7% in *S. flexneri* and 100% in *S. sonnei*). MDR phenotype was observed in 10/41 (24.4%) of *Shigella* isolates. Of these 10, 8 (80%) were resistant to CTX, CTR, CAZ, AMP, SXT, and ATH. Microbroth dilution results showed that the MIC of ATH varied between 8 and 64 μg/ml (data not included). No evidence of resistance to MRP or AMK was observed among *Shigella* isolates. ESBL phenotype was found only in one *Salmonella* isolate representing 7.1% of cases. Of all the *Salmonella* cases, 35.7% showed resistance to ATH. This pathogen showed no resistance, however, to MRP, CIP, AMK, or GM. All *C. jejuni* isolates were resistant to CIP, TET, and NA. Out of the seven *C. jejuni*, five

TABLE 4. ANTIMICROBIAL RESISTANCE PATTERNS OF DIARRHEAGENIC *ESCHERICHIA COLI*, *SHIGELLA* SPP., AND *SALMONELLA* SPP. ISOLATED FROM CHILDREN WITH ACUTE DIARRHEA ($N = 103$)

Pathogenic bacteria (number of isolates)[a]	*Antibiotic resistance: number of resistant isolates (%)*									
	ESBL+	*MRP*	*CAZ*	*CTX*	*CTR*	*CIP*	*AMK*	*AMP*	*SXT*	*GM*	*ATH*
Diarrheagenic *E. coli*[47]	32 (66.7)	0 (0)	28 (58.3)	32 (66.7)	32 (66.7)	15 (31.3)	16 (33.3)	45 (93.8)	37 (77.1)	22 (45.8)	NT
EAEC[26]	22 (84.6)	0 (0)	19 (73.1)	22 (84.6)	22 (84.6)	7 (26.9)	7 (26.9)	26 (100)	22 (84.6)	13 (50)	NT
EPEC[12]	4 (33.3)	0 (0)	3 (25)	4 (33.3)	4 (33.3)	4 (33.3)	2 (16.7)	9 (75)	8 (66.7)	2 (16.7)	NT
EIEC[6]	3 (50)	0 (0)	3 (50)	3 (50)	3 (50)	3 (50)	5 (83.3)	6 (100)	3 (50)	6 (100)	NT
ETEC[4]	3 (75)	0 (0)	3 (75)	3 (75)	3 (75)	1 (25)	2 (50)	4 (100)	4 (100)	1 (25)	NT
Shigella spp.[41]	22 (53.7)	0 (0)	16 (39)	22 (53.7)	23 (56.1)	6 (14.6)	0 (0)	39 (95.1)	30 (73.2)	3 (7.3)	9 (21.9)
S. flexneri[33]	18 (54.5)	0 (0)	12 (36.4)	18 (54.5)	19 (57.6)	5 (15.1)	0 (0)	33 (100)	22 (66.7)	3 (9.1)	6 (18.2)
S. sonnei[8]	4 (50)	0 (0)	4 (50)	4 (50)	4 (50)	1 (12.5)	0 (0)	6 (75)	8 (100)	0 (0)	3 (37.5)
S. enterica sub I[b,14]	1 (7.1)	0 (0)	1 (7.1)	2 (14.3)	1 (7.1)	0 (0)	0 (0)	2 (14.3)	2 (14.3)	0 (0)	5 (35.7)

A minimum of two independent experiments were performed to identify an ESBL phenotype or the resistant phenotype of the isolated pathogen against each antibiotic.
[a]Percentage of children from whom the indicated pathogen was isolated or detected.
[b]Subspecies enterica.
ESBL, extended-spectrum beta-lactamase; MRP, meropenem; CAZ, ceftazidime; CTX, cefotaxime; CTR, ceftriaxone; CIP, ciprofloxacin; AMK, amikacin; AMP, ampicillin; SXT, trimethoprim/sulfamethoxazole; GM, gentamicin; ATH, azithromycin. NT, not tested.

isolates were also resistant to AMP, rendering them MDR. No evidence of resistance to MRP, GM, or ATH was observed in *C. jejuni* isolates.

Discussion

Diarrhea in children remains an important public health concern in Iran. Although diarrhea is mostly self-limiting and does not require intervention, antimicrobial therapy is indicated for some non-viral diarrhea to shorten the illness and shedding of bacteria.[31] In Iran, however, it is common to employ an antibiotic therapy, often without ordering any stool cultures. Therefore, collecting local information on the epidemiological status of common enteric pathogens that cause acute diarrhea as well as information on their patterns of resistance to antibiotics is of considerable value not only for the development of local treatment guidelines but also for warning the national health system policy makers about the importance of resistance issue.

This cross-sectional study that covered a 7-month span was conducted on 269 outpatient children younger than 18 years who had acute diarrhea. The objectives were to determine the prevalence of four common bacterial enteric pathogens associated with diarrhea and the patterns of antibiotic resistance, as well as ESBL production in isolated pathogens. Using a combination of traditional microbiological techniques and molecular tests, we detected at least one bacterial pathogen in 40.9% (110/269) of cases of diarrhea. In the majority of cases (89.9%), one enteric pathogen was identified, and in the remainder of cases (10.1%), more than one pathogen was isolated, making it a challenge to identify the agent that causes the diarrhea. Our isolation rate falls within the range of 27.9–55.1% reported by similar studies done in Iran,[4,10,15] and exceeds those done in other countries (4.8–26.8%).[13,32–34]

In this study, DEC (48 isolates; 43.6%) and *Shigella* species (41 isolates; 37.3%) turned out to be the most prevalent etiological agents causing acute diarrhea. This was in agreement with the findings in previous studies carried out in Iran, in which DEC and/or *Shigella* species were the most common bacterial enteric pathogens (30.4–54%).[4,10,19,24] However, there is a recent report from Iran where EPEC and *Salmonella* spp. had been isolated most frequently in diarrhea cases.[35] The *Shigella* isolation rate in our study was much higher than that from studies carried out in other Asian countries as well as developed countries (1–5%).[36,37] Our result indicates that shigellosis is still an important public health problem in Iran and we should bring more awareness to safety of food, water, and sanitary condition to control and prevent the spread of shigellosis. In developing countries, the *S. flexneri* has been reported as the most common serotype, counting for up to 44.5–80% of all *Shigella* spp.[4,37–40] According to some reports from Iran, over a 3-year period (*i.e.*, 2002–2005),[10,41,42] an epidemiological change occurred in the serotype of *Shigella* species, with *S. sonnei* predominating. In line with other reports, however, *S. flexneri* appeared in our study as the most common species of all *Shigella* spp., responsible for 80.5% (33/41) of cases. Despite our finding, the reason for the serotype shifting during that particular period remains unknown.

With respect to the prevalence of the other species we isolated, *Salmonella* (14 isolates; 12.7%) and *C. jejuni* (seven isolates; 6.4%) were less common than DEC and *Shigella* species. The result is consistent with the results obtained by two previous reports during 2008–2009 from Iran, in which

the prevalence of *Salmonella* spp. and *Campylobacter* spp. were 7.6–13.8% and 5.4–10.8%, respectively.[4,10] There are few reports of *Campylobacter* spp. causing diarrhea in Iran, probably due to technical problems involved in the isolation of this strain.

In our study, enteric bacterial pathogens were not detected in 159 of the 269 stool samples (59.1%). This could be explained by the limitations of this study. To rule out viral diarrhea, we only included diarrheal samples in which WBC was present. Nonetheless, there is the possibility for other enteric pathogens (*e.g.*, parasitological agent.), which were not included in our diagnostic panel, to have been present. Another limitation is that patients might have taken antibiotics before sampling. There is a widespread tendency among clinicians in Iran to start antibiotic therapy in the case of acute diarrhea before the arrival of the results of a stool culture or even without ordering one. Accordingly, despite the lack of information on the probable use of antibiotics by the patients before taking the samples, we believe that a big part of our culture-negative stool samples is due to this reason.

Overall, more than 90% of tested DEC and *Shigella* isolates were resistant to AMP, and more than 70% to SXT. When compared to previous reports from Iran, a decrease in resistance to SXT and an increase to AMP are evident.[41,42] It can be concluded that these two antibiotics still are not appropriate for empirical therapy of shigellosis in Iran as reported previously.[41–43] In comparison to previous studies from Iran, in which the range of CTX and CTR-resistant DEC have been reported as 5.8–50.7% and 11.76–42.9%, respectively, our study showed a higher rate of resistance to third-generation cephalosporin in the case of DEC isolates (66.7%).[15,19,43] We also found a high level of CTX (53.7%) and CTR (56.1%) resistance among *Shigella* species isolates, which is not consistent with previous reports,[41,42] where no evidence of resistance to CTX and CTR had been observed. Resistant phenotype was confirmed through microbroth dilution method (data not included).

The common practice in Iran of recommending, on an empirical basis, oral cefixime for outpatient and parenteral CTR for inpatient treatment of acute diarrhea in children could be a possible explanation for high-level resistance to third generation of cephalosporins. For this reason, it is essential that antimicrobial susceptibility be regularly monitored so that an effective treatment can be recommended against locally isolated *Shigella*.

According to antibiotic susceptibility testing by disc diffusion method in this study, 21.9% of *Shigella* isolates were resistant to ATH for which microbroth dilution was employed to confirm resistance and determine the MIC of ATH, which was measured to be between 8 and 64 μg/ml (data not included). When we compare this to a previous report from Iran, resistance to ATH in *Shigella* isolates is much lower. Previously, resistance to ATH was reported as high as 70.4% in Isfahan.[44] We think that the reason for this difference is the use of nonstandard, home-made antibiotic disc for antibiotic susceptibility testing in that study. It is noteworthy to mention here that when running antibiotic susceptibility tests, it is critical to use standard discs and the right drug concentration in order for the results to be comparable. Ignoring this requirement can lead to unreliable indices for resistance. The American Society of Pediatrics has suggested ATH as a drug of choice for the treatment of resistant shigellosis cases.[31] In our study, MDR phenotype was observed in 24.4% (10/41) of *Shigella* isolates. In most (80%) of these cases, resistance was observed to not only CTX, CTR, CAZ, AMP, and SXT but also to ATH. With this in mind, we could not propose ATH as an alternative drug in the cephalosporin-resistant *Shigella* isolates.

Our results indicate MDR to AMP, CIP, NA, and TET in five of the seven *C. jejuni* isolates (71.4%). Also, in Iran, *C. jejuni* and *C. coli* have been shown resistant to CIP, TET, and NA isolated from different animal sources.[45–47] This resistant *Campylobacter* is capable of being transferred from animal products to humans, which could account for a high rate of resistance to CIP, TET, and NA shown by *C. jejuni* isolated in our study.

The study reports a high-level ESBL production of *Shigella* spp. and DEC. The high rate of ESBL phenotype in the two most prevalent agents causing acute diarrhea in Shiraz calls for a reconsideration of the current choice of antibiotics for acute diarrhea in children. As in our study, the diarrhea-causing pathogens showed high susceptibility rate to CIP (68.7% of DEC and 85.4% of *Shigella* isolates), one possibility of change to therapy protocols would be replacing the currently used CTR with CIP, which had been recommended by WHO since 2005 as the drug of choice for the treatment of shigellosis for every patient irrespective of their age.[48] This option, however, would need to undergo a systematic clinical study. According to this study, another alternative drug for the treatment of acute diarrhea caused by DEC and *Shigella* strains could be MRP with 100% susceptibility rate. However, since MRPs are antimicrobial agents used to treat serious infections in case an organism is resistant to primary antibiotic choice and in the case of nosocomial and mixed bacterial infections, we could not propose it as an alternative drug for treatment of acute diarrhea.

Finally, results are suggestive of the need to initiate a nation-wide, continuous surveillance to discover not only the causative agents of acute diarrhea but also the antimicrobial resistance pattern of the detected pathogens as well. Such epidemiological information would have the potential to contribute to the development of a more vigorous program to control the overuse of antibiotics, in turn leading to more informed decisions about the protocols to adopt for the treatment of acute diarrhea.

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Disclosure Statement

No competing financial interests exist.

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