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ORIGINAL ARTICLE

Molecular diagnosis and antimicrobial resistance pattern of *Shigella spp.* isolated from patients with acute diarrhea in Tehran, Iran

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ABSTRACT

Aim: To evaluate antimicrobial resistance pattern of Shigella spp. isolated from patients with acute diarrhea in Tehran, Iran.

Background: Infectious diarrheal diseases caused by Shigella spp. are significant health concern world wide. They are responsible for considerable morbidity and mortality especially in developing countries.

Patients and methods: A total of 1120 fecal samples from patients with acute diarrhea from May 2003 to May 2005 were investigated to evaluate antimicrobial susceptibility patterns of predominant isolates from different hospitals in Tehran, Iran. Identification of isolates was carried out according to standard methods.

Results: Among isolated enteropathogens, Shigella spp. were found in 14%. Shigella sonnei was commonest (56.1%) followed by Shigella flexneri, boydii and dysenteriae that were found in 30.6%, 8.3% and 5.1% of isolates, respectively. Of all isolates, 66.3% were detected in patients less than 5 years old. Resistance pattern was as follow: tetracycline 95.5%, ampicillin 51.5% and trimethoprim-sulfamethoxazole 91.7%. None of the isolates were resistant to ciprofloxacin, ceftriaxone and ceftazidime. An interesting finding in our study was a high degree of multidrug resistance to 3 and more antibiotics among isolates 91%.

Conclusion: In conclusion, Shigella species isolated from acute diarrhea in Iran have a high degree of resistance to commonly used antibiotics. The emergence of multidrug-resistance demands continues monitoring of susceptibility pattern of Shigella isolates. The changing of resistance pattern to common antibiotics in Iran indicates that designing a monitoring system for detection of antimicrobial resistance and guidelines for the appropriate use of antibiotics are urgently needed.

Keywords: *Shigella, Antimicrobial resistance, Diarrhea, Iran*.

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INTRODUCTION

Infectious diarrheal diseases caused by Shigella spp. are significant health concern world wide. They are responsible for considerable morbidity and mortality especially in developing countries (1, 2). It has been estimated that annually 1.1 millions of all deaths are attributable to shigellosis through the world. The diarrheal diseases in children are responsible for 25% of all deaths (1). The annually number of shigellosis is approximately 164.7 cases world wide while 163.2 millions of the cases are in developing countries (3). Shigella is the most important bacterial causes of diarrhea in children less than 5 years old. Shigella is divided to four species, classified on the basis of biochemical and

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serological differences including S. dysenteriae (Group A), S. flexneri (Group B), S. boydii (Group C), and S. sonnei (Group D) (3,4).

The predominant serogroup of Shigella is associated with the level of socioeconomic development. Shigella flexneri is the main serogroup found in developing countries (median 60% isolates) with sonnei being the next most common (median15%). Shigella dysenteriae, which is found most often in south Asia, and sub-Saharan Africa, and Shigella boydii occur with equal frequency (median6%) (5). S. boydii and S. sonnei are associated with mild short illness whereas infections caused by S. flexneri are more severe and last longer. Finally, S. dysenteriae causes the most severe illnesses associated with a high mortality rate (6).

Despite other enteric infections, shigellosis is one of acute diarrheal diseases for which antimicrobial therapy is prescribed (7). Over the past decades, Shigella spp. have shown a pattern of steadily increasing resistance to antibiotics and, strains of Shigella have progressively become resistant to most of the widely used antimicrobial agents and even newer antibiotics (8).

In the developing world, where the rate of diarrheal diseases is highest and indiscriminate use of antimicrobial agents is common, antimicrobial resistance in enteric pathogens is of utmost importance (7). Therefore, determination of antibiotic susceptibility of Shigella strains will be useful to administer the best antibiotic. The recovery rates of Shigella spp. are usually difficult to obtain in that these are very fastidious organisms and need special conditions to growth. These conditions usually take 48 to 72 h to provide (9). The present study was designed to investigate the antimicrobial resistance pattern of Shigella spp. based on biochemical, microbiological, and molecular diagnostic techniques in a group of Iranian patients with acute diarrhea.

PATIENTS and METHODS

Sample collection and bacteriologic isolation:

Sampling was performed during May 2003 to May 2005 from 6 different hospitals in Tehran. Fecal samples from patients with acute diarrhea were transported to the laboratory of National Research Department of Foodborne Diseases in Research Center for Gastroenterology and Liver diseases (RCGLD) in Tehran, in Cary-Blair and PBS transport media on ice packs and processed within the first hours following. Samples were cultured directly on MacConkey agar, Xylose–Lysine, De carboxylate agar and Salmonella–Shigella agar and incubated at 37°C over night.

Serological typing:

Colonies, morphologically resembling *Shigella* species, were further identified by biochemical reactions according to the standard methods (10) and confirmed by slide agglutination test using commercially available antisera from Mast Group Ltd.(MAST House, Derby Road, Bootle, Merseyside, L201EA, UK).

PCR Amplification:

DNAs were extracted from the organisms as described above (11). *Ipa*H gene on the chromosome (also present on the plasmid) was tested by polymerase chain reaction (PCR). A 2µl aliquot of this suspension was added to 22µl of PCR mixture (50 mM KCl, 10 mM Tris- HCl (PH: 8.3), 1.5mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate and 0.6U of Taq DNA polymerase and 1µl of primer mix 1 or 2 containing the primer at the concentration of 10ng/µl each. All samples were amplified in a programmable Thermocycler (Eppendorf AG 2233, Hamburg, Germany) for the following cycling parameters: 95^{o} C for 2 min to denature DNA, then 30 cycles of 30S at 94^{o} C, 30S at 42^{o} C to anneal the primers of the IpaH gene (Forward: 5'-CCTTTTCCGCGTTCCTTG A-3' and Reverse: 5'-CGGAATCCGGAGGTATTGC-3') (12) and 20 S at 72^{o}C, and finally, one prolonged

extension at 72°C for 10 min. The PCR product bands of 620 base pairs were separated by electrophoresis through agarose gel 1.5% in 1X TBE buffer. DNA fragments visualized by ethidium bromide staining and photographed under UV light illumination.

Antibiotic susceptibility testing:

Isolates were tested for susceptibility to erythromycin (E, 10μg), cefalothin (CF, 30μg) tetracycline (TE, 30μg), ampicillin (AM, 10μg), ceftazidime (CAZ, 30μg), nalidixic acid (NA, 30μg), gentamicin (GM, 10μg), amoxicillin–clavulanic acid (AMC, 30μg), trimethoprim-sulfamethoxazole (SXT, 300μg), cefixime (CFM, 5μg), ceftriaxone (CRO ,30μg), chloramphenicol (C , 30μg), ampicillin–sulbactam (AS, 20 μg) and ciprofloxacin (CP, 5μg) by Kirby–Bauer disk diffusion method and the resistance break – point used were those recommended by the National Committee for Clinical Laboratory Standard (NCCLS)(13). Results were recorded as either sensitive or resistant. *Staphylococcus aureus* (ATCC 25923) American Type Culture Collection (ATCC), and *Escherichia coli* ATCC 25922 were used as quality control.

Statistical analysis:

All data were analyzed by SPSS software version 11.0 (SPSS Inc, Chicago, IL, USA). Comparative statistics were calculated using the two-tailed $\chi 2$ test and Fisher's exact test, when appropriate. A p-value of ≤ 0.05 was considered to be significant.

RESULTS

From April 2004 to September 2005, the laboratory of RCGLD received and tested 1120 stool samples from patients with acute diarrhea. Among all enteropathogens, Shigella was commonest and isolated from 157(14.1%) samples. According to the serological tests, 88(56.1%) were identified as a S. sonnei and S. flexneri, S. boydii and S.dysenteriae were observed in 48(30.6%), 13(8.3%) and 8(5.1%), respectively.

Among cases, 66.3% were less than 5 years old whereas 11.5% were less than 1 year old. The prevalence of Shigella spp. isolated from the patients and their age distribution are shown in Table1. The isolation rate of Shigella spp. in spring, summer, fall and winter was 14.1 %, 51.6%, 19.8% and 14.7%, respectively. Of 88 S. sonnei and 46 S. flexneri isolates, 46(52%) and 22(25%) were isolated in the summer respectively (Figure1).

The antimicrobial resistant patterns of 14 different commonly used antibiotics were determined. Shigella isolates were determined by the Kirby-Bauer method. Result revealed that Shigella isolates were resistant to one or more agent 100 % while 91.7% were multidrug-resistant. Shigella strains showed high rates of resistance to tetracycline (95.5%) and sulfamethoxazol trimethoprim (91.7%). Also more than 90% of Shigella strains were susceptible to cefixime, ceftriaxone, ceftazidime, nalidixic acid, gentamicin, ciprofloxacin and ampicillin-sulbactam (Table2).

Table 1. *Age distribution of Shigella spp. isolated from diarrheal patients in Iran*

Pathogen	N (%)	0-12M	13-24M	25-36 M	37-48 M	49-60 M	>60M
S. sonnei	88(56.1*)	8(9.2)	12(13.6)	15(17)	18(20.4)	8(9.2)	27(30.6)
S.flexnery	48(30.6)	5(10.4)	3(6.3)	10(20.8)	5(10.4)	7(14.6)	18(37.5)
S. boydii	13(8.2)	0	0	6(46.1)	0	2(15.3)	5(38.6)
S. dysenteriae	8(5.1)	5(62.5)	0	0	0	0	3(37.5)

M: Month, * percentage

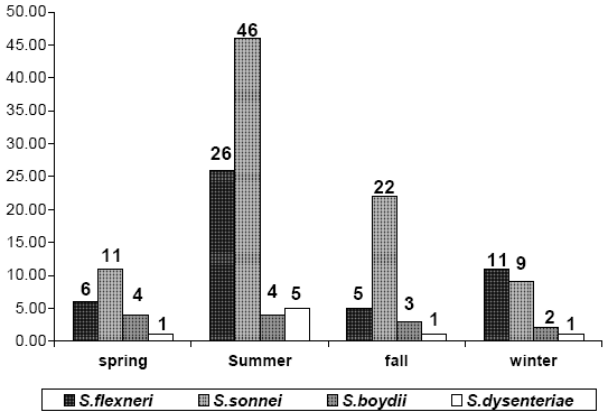

Figure 1. *Number of Shigella spp. isolated from diarrheal patients in different seasons in Iran*

Totally 85(96.5%) of Shigella flexneri strains were resistant to tetracycline and trimethoprim-sulfamethoxazole and 66(75.1%) to erythromycin.

All S.sonnei and S.flexneri strains were susceptible to chloramphenicol, ceftazidime and gentamicin. Meanwhile 97.4% of the isolates showed susceptibility to nalidixic acid and 99.3% to ampicillin-sulbactam. It was observed that 143(91%) strains were resistant to three or more antibiotic distributed into 25 distinct patterns. Of these, 6 strains were resistant to 6 antibiotics. The most frequent multidrug resistant pattern among S. sonnei was to erythromycin, tetracycline, sulfomethaxazol (21.6%) followed by erythromycin, tetracycline, ampicillin and sulfomethaxazol (18.18%).

Forty-eight strains of Shigella flexneri showed a high proportion of resistant strains to tetracycline (95.8%), sulfamethoxazol-trimethoprim (87.5%) and erythromycin (66.4%). Among S. flexneri strains, 8 were resistant to 6 antibiotics, however, we found 1 strain which was resistant to the following 7 antibiotics: erythromycin, cefixime, tetracycline, ampicillin, sulfamethoxazole-trimethoprim, ampicillin-sulbactam and chloramphenicol. The most frequent multidrug-resistant pattern among S. flexneri strains was the combined resistance to (erythromycin, tetracycline, ampicillin, sulfamethoxazol-trimethoprim, and ampicillin-sulbactam) which was found in 7(14.5%) isolates.

All the Shigella boydii strains were susceptible to ceftazidime, gentamicin, ceftriaxone, ciprofloxacin, and ampicillin-sulbactam while 74.9% of Shigella boydii strains were resistant to 3 or more antibiotics at the same time. Resistance to 6 antibiotics was also observed. Meanwhile Shigella boydii showed high susceptibility to chloramphenicol (92.3%), cefixime (61.4%) and nalidixic acid (84.6%). Despite, 84.6% were resistant to tetracycline (Table 2). All Shigella dysenteriae strains were resistant to tetracycline. Moreover Shigella dysenteriae were susceptible to ceftriaxone, ceftazidime, nalidixic acid, gentamicin, ciprofloxacin, chloramphenicol and ampicillin-sulbactam (100%).

Table 2. *Antimicrobial resistance of Shigella spp. isolates from acute diarrhea in Iran*

	E	CF	CFM	CRO	TE	SAM	AM	CAZ	SXT	NA	AMC	GM	C	CIP
S. sonnei	75	29.5	1.1	0	96.5	56.8	0	96.5	2.3	30.6	0	9	0	1.2
S. flexneri	66.4	8.3	0	0	95.8	47.9	0	87.5	0	54.1	4.2	47.9	0	0
S. boydii	84.6	38.4	0	0	84.6	38.4	0	76.9	15.4	61.5	0	7.6	0	0
S. dysenteriae	75	12.5	0	0	100	37.5	0	87.5	0	12.5	0	0	0	0

AM-ampicillin; SXT-trimethoprim-sulfamethoxazole; C-chloramphenicol; TE-tetracycline; CF-cephalothin; CRO-ceftriaxone; CIP-ciprofloxacin; GM-gentamicin; E-erythromycin; NA-nalidixic acid; CAZ-ceftazidime; SAM-ampicillin sulbactam; AMC-amoxicillin clavulanic acid; CFM-cefixime

DISCUSSION

Shigellosis is a world wide health concern especially in developing countries with poor sanitation, lack of personal hygiene and use of contaminated water supplies (14-16). Like many other developing countries, Shigella plays a significant role in the disease burden of Iran (17,18).

In this study, the frequency of dysentery among patients with acute diarrhea (9.6%) was more or as the same as other reports from developing countries (17,20,21).

In developing countries, S. flexneri is the predominant Shigella spp. recovered from patients with acute diarrhea and represents 50-70% of all Shigella isolates. Our results revealed the predominant prevalence of Shigella sonnei in children as well as in adults (17,18). Our result is in agreement with developed countries, even though, Iran is a developing country (22,23). S. sonnei is the predominant species and is more common in children than in adults. Like other countries the isolation rate of Shigella spp. was highest in summer (51.5%).

Multidrug-resistant patterns in bacterial pathogens are now common in developing countries such as Iran (24). Our results of antimicrobial susceptibility tests described relatively higher numbers of multidrug-resistant isolates compared with prior reports from Iran (17,25). It was clarified that 143(91%) strains were resistant to three or more antibiotics distributed into 25 distinct patterns. Interestingly, six strains were resistant to 6 different antibiotics while one strain was resistant even to 7 antibiotics. This could be explained by the fact that there are selective pressure causing the appearance and reemergence of specific strains (25). It has been reported that antimicrobial resistance gene can be readily transmitted between comensal enterobacteriaceae and enteropathogens in-vivo and in-vitro (26).

Shigella has been becoming resistant to most antibiotics commonly used in the treatment of diarrhea.

According to the susceptibility of the majority of Shigella spp. to cefixime, ciprofloxacin, ceftriaxone and nalidixic acid in this study, nalidixic acid is recommended as the drug of choice for shigellosis in both adults and children. Based on our findings, we conclude that Shigella spp. can be considered as an important etiological agent of diarrhea, with a high rate of drug resistance in the region.

Furthermore, our findings showed that third generation cephalosporins should be kept in reserve, only for the treatment of drug-resistant non-responsive cases of acute gastroenteritis. Notably, co-trimoxazole, tetracycline, and ampicillin had no reasonable role in the empirical treatment of gastroenteritis. These drugs could be replaced with other antibiotics such as quinolones.

Determining the prevalence rate of diarrheal pathogens should save the way for better control of the disease in the country. Continued vigilance of the safety of food, health education of food handlers, and close attention to hygiene and sanitary conditions can provide an effective barrier against the spread of shigellosis. The antimicrobial resistance may be as a result of inappropriate and wide use of different antibiotics to treat infection. The changing patterns of resistance to common antimicrobial agents in Iran indicates that designing a surveillance system for antimicrobial resistance and the introduction of integrated guidelines for the appropriate use of antibiotics are urgently needed.

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