



Antibiotic Sensitivity Patterns and Molecular Typing of *Shigella sonnei* Strains Using ERIC-PCR

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Abstract

Background: *Shigella sonnei* is considered as a major cause of diarrheal disease in both developing and developed countries. Iran is one of the endemic areas of shigellosis. The present study was undertaken to investigate the antibiotic susceptibility and genetic relatedness of *S. sonnei* strains isolated from pediatric patients in Tehran, Iran.

Methods: The study included all *S. sonnei* strains isolated from pediatric patients with diarrhea admitted to several hospitals in Tehran, Iran, during 2008-2010. *Shigella* spp. strains were recovered from patients using standard microbiological methods. *S. sonnei* strains were further studied by antimicrobial susceptibility testing and Enterobacterial Repetitive Intergenic Consensus (ERIC) - PCR analysis.

Results: Eighty nine *Shigella* isolates were isolated. *S. sonnei* was the most prevalent *Shigella* species (60.7%) followed by, *S. flexneri* (31.5%). Eleven antimicrobial resistance patterns (R₁-R₁₁) were identified among *S. sonnei* isolates. The majority of the strains were resistant to trimethoprim-sulfamethoxazole, tetracycline and streptomycin. All isolates were susceptible to ciprofloxacin, ceftizoxime and chloramphenicol. All strains were typable by ERIC-PCR. Five ERIC-PCR patterns (E₁-E₅) were found among *S. sonnei* isolates; however the half of the isolates was clustered in E4 pattern.

Conclusion: The antibiotic resistance rates are increasing among *S. sonnei* strains. Moreover, a predominant clone or limited clones of *S. sonnei* were responsible for shigellosis caused by this *Shigella* species in pediatric patients in Tehran, Iran.

Keywords: *Shigella sonnei*, Antibiotic resistance, ERIC-PCR

Introduction

Shigellosis is among the most common causes of bacterial diarrheal disease in both developing and developed countries. *Shigella* consists of four serogroups including serogroup A (*S. dysenteriae*), serogroup B (*S. flexneri*), serogroup C (*S. boydii*) and serogroup D (*S. sonnei*). It is one of the major causes of morbidity in children with diarrhea in Iran (1, 2). *S. sonnei* is the major cause of shigellosis in industrialized countries (3). It has been recently reported it as the prevalent *Shigella* serotype in Iran (4, 5).

Treatment using adequate antibiotics is effective for shigellosis particularly for the children and immunosuppressed patients because it may shorten the clinical course of the disease, reduce the risk of transmission and prevent potentially lethal complications. However, resistance to commonly used antibiotics is increasing among *Shigella* spp. worldwide (6, 7). The antibiotic resistance among *Shigella* spp. is increasing in Iran (8-10).

In the recent years, the conventional bacterial typing methods such as antimicrobial resistance pat-

tern, bacteriophage typing, or serotyping have been replaced by molecular techniques such as ribotyping, pulsed-field gel electrophoresis (PFGE), and PCR-based methods (1,11,12). An Entero-bacterial Repetitive Intergenic Consensus (ERIC) sequence is an imperfect palindrome of 127 bp. ERIC sequences have been found only in intergenic regions, apparently only within transcribed regions. PCR-mediated genomic fingerprinting based on ERIC sequences has been found to be useful for subtyping Gram-negative enteric bacteria and differentiation of their strains (13).

The present study was conducted to determine the antimicrobial susceptibility and genetic relationship among *S. sonnei* strains isolated from pediatric patients in Tehran, Iran.

Material and Methods

Bacterial strains

The study included all *S. sonnei* strains isolated from pediatric patients with diarrhea who were admitted to several hospitals in Tehran, Iran, during 2008-2010. A single specimen was obtained from each patient, and rectal swabs were collected from patients on the day of admission at the hospital. When the isolates were identified as *Shigella* by the conventional methods (14), these were serotyped using slide agglutination with specific antisera (MAST Group LTD, Merseyside, UK).

All ethical issues were considered. The name and characters, personal information and even patients' illnesses and their medical information remained secret and the life, health, dignity, integrity, rights to self-determination, privacy, and confidentiality of personal information of research subjects were protected in this study.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed according to Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (15).

The following antimicrobial agents were tested: ampicillin (AM,10 μ g), amoxicillin-clavulanic acid (AMC 20+10 μ g), amikacin (AN 30 μ g), ceftazidime

(CAZ 30 μ g), cephalothin (CF 30 μ g), ceftriaxone (CRO 30 μ g), cefotaxime (CTX 30 μ g), kanamycin (K 30 μ g), nalidixic acid (NA 30 μ g), streptomycin (S 10 μ g), trimethoprim-sulfamethoxazole (SXT 23.75+1.25 μ g), tetracycline (TE 30 μ g), ticarcillin (TIC 75 μ g), tobramycin (TOB 10 μ g), gentamicin (GM 10 μ g), ceftizoxime (CT 30 μ g), ciprofloxacin (CP 5 μ g), chloramphenicol (C 30 μ g). *Escherichia coli* ATCC 25922 were used as control strain.

DNA preparation

One colony from overnight culture was inoculated into LB-broth (tryptone 1%, NaCl 1% and yeast extract 0.5%) and was grown with shaking overnight at 37°C. Bacterial DNA was extracted using the phenol-chloroform method. Extracted genome was dissolved in 50-100 μ l of TE buffer.

ERIC-PCR primers and PCR condition

ERIC-PCR was performed using the primers ERIC 1R (5'-ATGTAAGTCTCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') with minor modifications (16, 17).

The PCR reaction mixture (20 μ l) consisted of 10x reaction buffer [750 mM Tris-HCl (pH 8.8), 200 mM (NH₄)₂SO₄, 0.1% Tween20], a 250 μ M concentration of each dNTP, 20 pmol of primer ERIC 1R, 20 pmol of primer ERIC 2, 3.75 mM MgCl₂, 100 ng of template DNA and 2 U of *Taq* polymerase. The reaction mixture was denatured for 7 min at 95 °C and then subjected to 30 cycles of denaturation for 30s at 90 °C, annealing for 1 min at 52 °C, extension for 3 min at 65 °C, and a final extension for 16 min at 65 °C. Negative controls without template DNA were included in each run. Amplified products were resolved by electrophoresis of 5 μ l samples on 1% agarose gels in 1x TBE buffer at 70 V for 3 h and were visualized by ethidium bromide staining. The PCR patterns were visually compared and were considered to be identical on the basis of similar numbers and matching positions of all major bands. Small differences in bands were ignored. The type strain of *S. sonnei*, ATCC 9290, was also included in this study for the comparison.

Results

Of 950 patients with acute diarrhoea, 89 *Shigella* spp. strains were isolated which were distributed as following; *S. sonnei*, 54 (60.7%), *S. flexneri* 28 (31.5%), *S. boydii* 5 (5.6%) and *S. dysenteriae* 2 (2.2%). The season distribution of the isolated strains was winter, 10; spring, 17; summer, 38 and fall, 24. Of all patients, 57.4% were male and 42.7% were female. The ratio of males to females was 1.34. *Shigella* was isolated frequently from children under 5 years of age, who accounted for 62.9.7% of all isolates. About 35.1 % of all isolates came from persons aged 5-12 years, and 1.8% from persons aged over 12 years of age.

Results of further examination of *S. sonnei* strains showed that the majority of the isolates ($\geq 90\%$) were resistant to trimethoprim-sulfamethoxazole, tetracycline and streptomycin, 37% to ampicillin, 27.8% to nalidixic acid, 9.3% to amoxicillin-clavulanic acid and 11.3% tobramycin. The most of the isolates were susceptible to cephalothin, ticarcillin, cefotaxime, ceftriaxone, amikacin, ceftazidime, kanamycin and gentamicin. All strains were fully susceptible to ciprofloxacin, ceftizoxime and chloramphenicol. As shown in table 1, the majority of the isolates demonstrated multiple drug resistance profile, among which, 1.8% were resistant to 2, 57.4% to 3, 3.7% to 4, 16.7% to 5, 7.4% to 6, 1.8% to 9, 5.5% to 11 and 1.8% were resistant to 12 tested antibiotics. Eleven antibiotic resistance patterns (R_1 - R_{11}) were observed among the strains, R_1 (tetracycline/ streptomycin/ trimethoprim-sulfamethoxazole) was however found as most prevalent (51.8%) resistance pattern.

All strains were typable by ERIC-PCR. The number of ERIC-PCR bands produced for a given primer ranged from 10 to 15, with molecular sizes ranging from 100 to 3500 bp. ERIC-PCR analysis of the isolates resulted in five different patterns (E_1 - E_5) with 9-13 DNA bands (Fig. 1); however the half of the isolates was clustered in E_4 pattern. Table 1 shows the distribution of ERIC-PCR patterns. No similarity was observed in the ERIC-PCR patterns between clinical isolates and ATCC type strain 9290 (Fig. 1).

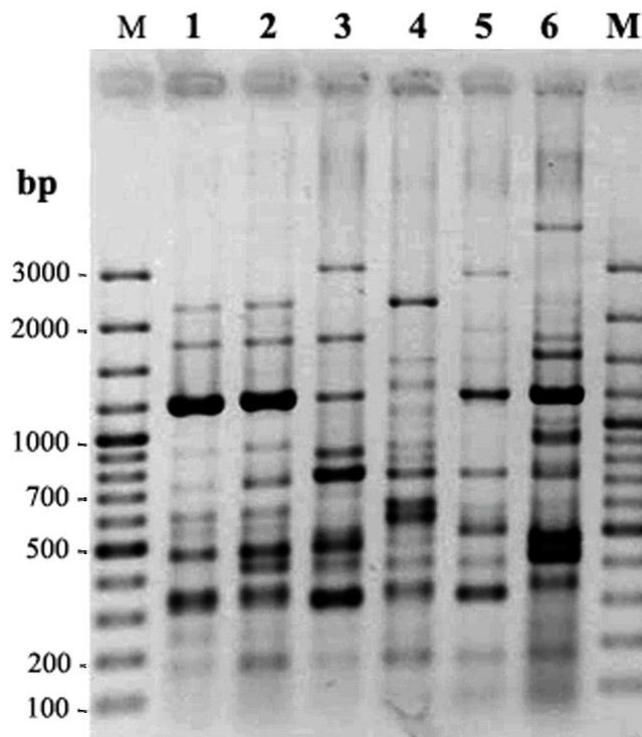


Fig. 1: ERIC-PCR patterns of *S. sonnei* isolates. Lanes 1-5 are representative patterns E_1 , E_2 , E_3 , E_4 and E_5 respectively. Lane 6 is E_s pattern from type strains ATCC 9290. Lane M is molecular size marker

Discussion

The infections caused by *Shigella* spp. has been increasing continuously and has turned into a prominent public health concern worldwide. *Shigella* spp. is one of the major causes of diarrheal disease among children in Iran (18).

Shigella strains were isolated frequently from children under 5 years of age, who accounted for 62.9.7% of all isolates. Previous studies have also demonstrated the fact that age can be a risk factor in shigellosis where children are in the high risk zone for *Shigella* infections (19). The typical seasonal increase in shigellosis occurred during the summer, with peak incidence in August that confirmed the warm months of the year can intensify the frequency of *Shigella* associated infections.

Table1: Characteristics of *S. sonnei* isolates included in the study

No. of isolates	Resistance pattern* (R ₁ -R ₁₁)	ERIC-PCR pattern
Type strains (ATCC 9290)	-	Es
1	AMC, NA, CAZ, AM, TIC, CTX, CRO, TE, S, SXT, CF (R ₄)	E1
2	AMC, NA, CAZ, AM, TIC, CTX, CRO, TE, S, SXT, CF (R ₄)	E1
3	NA, AM, TE, S, SXT (R ₂)	E1
4	NA, AM, TE, S, SXT (R ₂)	E1
5	AMC, NA, CAZ, AM, TIC, CTX, CRO, TE, S, SXT, CF (R ₄)	E1
6	-	E2
7	NA, AM, TE, S, SXT (R ₂)	E4
8	NA, AM, TE, S, SXT (R ₂)	E1
9	NA, AM, TE, S, SXT (R ₂)	E2
10	NA, AM, TE, S, SXT (R ₂)	E3
11	NA, AM, TE, S, SXT (R ₂)	E1
12	AMC, NA, CAZ, AM, TIC, CTX, CRO, TE, S, SXT, GM, CF (R ₇)	E1
13	NA, AM, TE, S, SXT (R ₂)	E1
14	AM, TE, S, SXT (R ₉)	E5
15	NA, AM, TE, S, SXT (R ₂)	E4
16	TOB, TE, SXT (R ₁₁)	E4
17	AM, TOB, AN, TE, S, SXT (R ₃)	E4
18	AM, TOB, AN, TE, S, SXT (R ₃)	E4
19	TE, S, SXT (R ₁)	E4
20	AM, TOB, AN, TE, S, SXT (R ₃)	E4
21	TE, S, SXT (R ₁)	E4
22	TE, S, SXT (R ₁)	E4
23	AMC, AM, TOB, TIC, CTX, CRO, TE, S, SXT (R ₈)	E4
24	AM, TOB, AN, TE, S, SXT (R ₃)	E4
25	NA, S, SXT (R ₅)	E4
26	TE, S, SXT (R ₁)	E4
27	TE, S, SXT (R ₁)	E4
28	NA, S, SXT (R ₅)	E4
29	TE, S, SXT (R ₁)	E4
30	-	E2
31	TE, SXT (R ₁₀)	E2
32	TE, S, SXT (R ₁)	E4
33	TE, S, SXT (R ₁)	E2
34	TE, S, SXT (R ₁)	E2
35	TE, S, SXT (R ₁)	E1
36	TE, S, SXT (R ₁)	E1
37	TE, S, SXT (R ₁)	E4
38	TE, S, SXT (R ₁)	E2
39	TE, S, SXT (R ₁)	E2
40	TE, S, SXT (R ₁)	E2
41	TE, S, SXT (R ₁)	E2
42	TE, S, SXT (R ₁)	E2
43	TE, S, SXT (R ₁)	E4
44	TE, S, SXT (R ₁)	E2
45	TE, S, SXT (R ₁)	E4
46	TE, S, SXT (R ₁)	E2
47	TE, S, SXT (R ₁)	E4
48	TE, S, SXT (R ₁)	E4
49	TE, S, SXT (R ₁)	E4
50	TE, S, SXT (R ₁)	E4
51	TE, S, SXT (R ₁)	E4
52	K, AM, TE, SXT (R ₆)	E4
53	TE, S, SXT (R ₁)	E4
54	TE, S, SXT (R ₁)	E4

*AM: ampicillin, AMC: amoxicillin-clavulanic acid, AN: amikacin, CAZ: ceftazidime, CF: cephalothin, CRO: ceftriaxone, CTX: cefotaxime, K: kanamycin, NA: nalidixic acid, S: streptomycin, SXT: trimethoprim-sulfamethoxazole, TE: tetracycline, TIC: ticarcillin, TOB: tobramycin, GM: gentamycin.

Assessment of antibiotic resistance patterns among *Shigella* isolates revealed that the antibiotic resistance rates have increased in comparison with previous reports published from Iran. For examples, when compared to a previous study in Tehran, the percentage of resistance against ampicillin, and nalidixic acid has increased from 10% and 8.3% to 37% and 27.8% respectively in our study (20). Moreover, to our knowledge, resistance to ceftriaxone and cefotaxime has not been reported in clinical strains of *S. sonnei* in Iran to date. However we found here 5 strains were resistant to these antibiotics.

Only one strain was resistant to kanamycin where also more than half of isolates showed intermediate resistance to this antibiotic which can be result in the probable increase in *Shigella* resistance to this antibiotic in the future. Moreover, in a ten-year study conducted by Ashtiani et al, in Tehran, Iran, *Shigella* spp. were found to show noticeably increasing resistance to kanamycin between 2001 and 2005 (21).

Fortunately resistance to ciprofloxacin and gentamicin is still low among our *Shigella* isolates. These finding is consistent with those reported by Vrints et al. who showed that the all *Shigella* isolates were sensitive to ciprofloxacin as well as gentamicin in a 18-years study from 1990 to 2007 in Belgium (22).

Subtyping using phenotypic methods is hindered by the homogeneity of circulating strains causing infectious diseases. Molecular typing of microbial strains helps us to accurately interpret epidemiological evolution of infectious diseases in the communities. Several different molecular methods have been applied to study the molecular epidemiology of *S. sonnei* isolates. PCR-based typing methods have been applicable to many organisms including *S. sonnei* and can be completed easily within a single day (18, 23).

ERIC-PCR is less laborious and time-consuming than other DNAbased typing techniques. This method has been widely used for the molecular typing of different bacteria in epidemiological studies, and its advantages and disadvantages are well known (24). However a few limited studies have previously evaluated the ERIC-PCR for mo-

lecular typing of *Shigella* strains. However as reported by Liu et al., this technique has been established to be a reliable and rapid genotyping approach with high discriminatory power for the differentiation of *Shigella* strains (25).

Here we used ERIC-PCR to study the genetic relatedness among endemic *S. sonnei* strains isolated from pediatric patients in Tehran, Iran. This technique differentiated the isolates into five different clusters (E₁-E₅). Using one pair of ERIC-PCR primers in a study carried out by Penatti et al, three genetic patterns were reported from *S. flexneri* and *S. sonnei* strains isolated from bacillary dysentery cases in Southeast Brazil (26).

This method has been also used for subtyping of epidemic *S. flexneri* strains in Iran previously. In a recent outbreak of shigellosis occurred among prisoners in Isfahan, ERIC-PCR showed to be a powerful method for molecular typing of *Shigella* strains. This technique differentiated the epidemic causative agent of outbreak from endemic and type standard strains and showed that a single clone of *S. flexneri* serotype 3a was responsible for the outbreak since all tested isolates had a single pattern (27). Kosek et al. also reported that this method was highly reproducible and could provide highly similar and supplementary data compared with serotyping regarding the transmission dynamics of shigellosis in the community studied (28). Otherwise, in another study for investigation of shigellosis outbreaks occurred in school children in Lungtan and Bade in Taoyuan county in northern Taiwan, this technique showed poor discriminatory power where ERIC-PCR analysis could not discriminate an epidemiologically unrelated strain from some outbreak strains. Epidemic *S. sonnei* strains could not be differentiated from type strain ATCC 9290 (29).

Here we also used type strain ATCC 9290 for the comparison; however ERIC-PCR was capable to differentiate endemic *S. sonnei* strains from this standard strain and from each other clinical strains distributed in different clusters.

Navia et al. used another PCR-based technique, in which the amplification of the regions between repetitive extragenic palindromic (REP) sequences gave a fingerprinting pattern valid for epidemio-

logical typing of different species of *Shigella* (30). Also in a study carried out on 60 *S. sonnei* strains isolated from children hospitalized at five hospitals in Tehran during 2003, a similar technique, known as Arbitrarily Primed PCR (AP-PCR), was evaluated for subtyping of endemic *S. sonnei* isoaltes. Only a single AP-PCR pattern was observed among all *S. sonnei* strains (23).

When reviewing the previous reports on the distribution of molecular types of endemic *S. sonnei* in Iran, our findings further confirm the involvement of our geographic area within an epidemiological global picture of dissemination of a limited number of well-defined clones of *S. sonnei*. The results obtained from our previous global project on 1,672 *S. sonnei* isolates obtained since 1943 from 50 countries including Iran revealed that three major *S. sonnei* groups were responsible for shigellosis caused by this serogroup in which two groups were globally spread (3).

Conclusion

Considering that more than half of isolates were clustered into E4, it is concluded that one predominant clone or limited clones of *S. sonnei* are responsible for shigellosis caused by this *Shigella* species in pediatric patients during our research period. We hope our finding could be helpful for further epidemiological surveillance of *S. sonnei* in our country in the future.

Continuous studies using more discriminating molecular methods is needed to be conducted in Tehran and other parts of Iran in order to determine molecular subtypes of *S. sonnei* and other *Shigella* species in the future.

Ethical Considerations

All ethical issues including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the author.

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