Drug resistance profiles and clonality of sporadic Shigella sonnei isolates in Ankara, Turkey

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Abstract

The aims of this study were to investigate drug resistance rates, types of extended spectrum beta lactamases (ESBLs), and molecular epidemiological characteristics of 43 Shigella sonnei isolates. Ampicillin-sulbactam, amoxicillin-clavulanate, chloramphenicol, and ciprofloxacin were the most active antibiotics. Five isolates harbored bla\textsubscript{SHV-12}, bla\textsubscript{TEM-1} and bla\textsubscript{CTX-M-15}. More than 90% of the isolates had an indistinguishable pulotype.

Key words: Shigella sonnei, extended spectrum beta-lactamase, plasmid profile, pulsed-field gel electrophoresis.

Shigellosis is a global human health problem caused by a group of bacteria called Shigella. There are four species of Shigella named S. sonnei, S. flexneri, S. dysenteriae, and S. boydii all of them capable of causing disease. In Turkey, S. sonnei is the most frequently reported Shigella species (56.5%), followed by S. dysenteriae (22.9%), S. flexneri (14.5%) and S. boydii (6.1%) (http://www.saglik.gov.tr/istatistikler/temel2005/tablo-65.htm.). Similarly, S. sonnei is the predominant serogroup in Ankara, the capital city of Turkey (Saran et al., 2013).

The increasing prevalence of antibiotic-resistant strains of Shigella has become a major concern worldwide. World Health Organization (WHO) recommends that all patients with bloody diarrhea should be treated with either ciprofloxacin or one of the following three drugs: pivmecillinam, azithromycin, and ceftriaxone (http://whqlibdoc.who.int/publications/2005/9241592330.pdf). However, there are many reports indicating fluoroquinolone resistant and extended spectrum beta-lactamase (ESBL)-producing Shigella isolates from certain countries in 2009-2011 years (Nandy et al., 2010; Sabra et al., 2009; Varghese and Aggarwal, 2011).

Molecular typing and antimicrobial susceptibility of S. sonnei isolates can provide very useful data for the management of both isolated cases and outbreaks (DeLappe et al., 2003; Liang et al., 2007; Na-Ubol et al., 2006). In this study, we investigated the rate of antimicrobial resistance of S. sonnei strains collected in Ankara, the capital city of Turkey, from 2004 to 2006 and analyzed ESBL-producing isolates by PCR and DNA sequencing. Clonal relationship among the S. sonnei isolates was assessed using pulsed field gel electrophoresis (PFGE) and plasmid profile analysis.

A total of 43 S. sonnei isolates were cultured from stool samples of patients with acute diarrhea. Isolates were collected at the Clinical Microbiology Laboratory of Gazi University Medical School Hospital in Ankara, Turkey during the three-year period between 2004 and 2006. Only one isolate was obtained from each patient. Twenty-three of the 43 patients (53.5%) were male. The ages of the patients ranged from 5 to 75 years, with a mean of 14.1. The isolates were identified to species levels using conventional biochemical tests and group-specific polyvalent antisera, followed by phase specific antisera (Denka Seiken UK Ltd., Derbyshire, UK). Susceptibilities of the isolates to

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ampicillin, chloramphenicol, ciprofloxacin, ampicillin-sulbactam, amoxicillin-clavulanate, ceftriaxone, cefixime, and trimethoprim-sulfamethoxazole were determined by disk diffusion method according to the criteria recommended by the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2010). ESBL production was determined by the double disk synergy method (Jarlier et al., 1988).

ESBL-producing S. sonnei isolates were tested for the presence of β-lactamase-encoding genes blaTEM, blaSHV and blaCTX-M by PCR. Approximately ten fresh colonies were suspended in sterile water, boiled for 10 min, and centrifuged for 2 min at 13000 x g. The supernatant was used as DNA source. Universal primers described previously for the β-lactamase-encoding genes were used (Ma et al., 2005; Sidjabat et al., 2009). The reaction mixture contained either 2.5 mM MgCl2 for blaSHV PCR or 1.5 mM MgCl2 for blaTEM and blaCTX-M PCR, each of the deoxynucleoside triphosphates at a concentration of 200 μM, 5 μL of 10 PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM (NH4)2SO4, 0.1% (v/v) Tween 20], 10 pmol of each primer, and 2.5 U of Taq DNA polymerase (Thermo Scientific-Fermentas Corporation, Vilnius, Lithuania) in a final reaction volume of 50 μL. Amplification of blaTEM and blaCTX-M was performed using the following conditions: initial denaturation at 94 °C for 4 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 4 min. The PCR products were purified with Dye-terminator cycle sequencing quick start kit (Beckman Coulter Company, Massachusetts, USA). Sequencing reactions were performed with the primers used for PCR reactions. Each sequence reaction mixture consisted of 3.5-5 μL purified amplicon, 5 pmol primer, and 4 μL of Dye terminator cycle sequencing quick start kit (Beckman Coulter Company, Massachusetts, USA). The sequence reaction was done as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles consisting of denaturation at 96 °C for 20 s, annealing at 55 °C for 20 s, and elongation at 60 °C for 4 min. The PCR products were purified with Dye-Terminator removal kit (Beckman Coulter Company, Massachusetts, USA) and 20 μL of purified product was sequenced in Beckman Coulter 8000 instrument. The resulting DNA sequences were compared with the GenBank sequence databases using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plasmid profiles analysis of the S. sonnei isolates was carried on plasmids obtained by using the alkaline-lysis method described by Kado and Liu (1981) with some modifications. Our modifications included culturing the isolates in ampicillin containing medium and exclusion of the phenol/chloroform purification step. Molecular sizes of plasmids were determined by using super coiled DNA ladder (Life Technologies, Carlsbad, CA, USA).

PFGE typing of the 43 S. sonnei isolates was performed by following the protocol of Durmaz et al. (2009). Briefly, bacterial cells were embedded into low melting agarose including sodium dodecyl sulfate. Cells in plugs were digested with lysozyme and proteinase K. Genomic DNA was restricted by 20 U of XbaI (Thermo Scientific-Fermentas Corporation, Vilnius, Lithuania). DNA fragments were separated by using a CHEF-DR II system (Bio-Rad Laboratories, Nazareth, Belgium). The DNA band profiles were analyzed with GelCompar software (version 3.0; Applied Maths, Sint-Martens-Latem, Belgium). A 1% band tolerance was used for comparison of DNA profiles. Clonal relationship among isolates was evaluated by using the criteria of Tenover et al. (1997).

All 43 isolates were confirmed as S. sonnei using group-specific polyvalent antisera and phase specific antisera. According to the serotyping results, 28 (65%) of the 43 isolates showed phase 1, eight (19%) showed phase 2, and seven (16%) showed both phase 1 and 2 profiles (+ 1). According to the antibiotic resistance profiles, four antibiotypes were designated as shown in Table 1. Since more than 83% of the isolates were classified in only one antibiotype (type 2), it was very difficult to discuss usefulness of the antibiotyping on our isolates collected during the three-year period. In this study, S. sonnei isolates showed the low rate of susceptibility against trimethoprim-sulfamethoxazole (5%). In agreement with our results, previous studies from Turkey and other countries also indicated small susceptibility rates (3.5-30%) (Akkali et al., 2008; Altun and Gur, 2011; Jafari et al., 2009; Saran et al., 2013; Zafar et al., 2009). The studies from Iran and Israel revealed 57% and 87% resistance to ampicillin, respectively (Ashkenazi et al., 2003; Jafari et al., 2009). In contrast to the results reported from those countries, low resistance was found in the current (12%) and previous studies (17% and 20%) from our country (Akkali et al., 2008; Altun and Gur, 2011). In accordance with some studies (Akkali et al., 2008; Altun and Gur, 2011; DeLappe et al., 2003), we found that all S. sonnei isolates were susceptible to ampicillin-sulbactam, chloramphenicol, and ciprofloxacin. However, other works have reported different resistance rates (9% to 100%) for chloramphenicol (Ashkenazi et al., 2003; Jafari et al., 2009).

In our study, resistance to ceftriaxone and cefixime was only detected in five (12%) ESBL-producing isolates. ESBL-positive S. sonnei isolates were also reported in Lebanon (8%) (Sabra et al., 2009), Kuwait (2.8%) (Jamala et al., 2010), China, and India (Izumiya et al., 2009). One of the ESBL-positive isolates carried blaSHV-12 and four harbored blaCTX-M-15 genes. To the best of our knowledge, this
Table 1 - Distribution of *S. sonnei* strains according to epidemiologic, phenotypic and molecular typing characteristics.

| Strain No | Isolation date (m/d/y) | Antibiotic susceptibility pattern | Phase type | PFGE type | Plasmid profile |
|-----------|------------------------|-----------------------------------|------------|-----------|-----------------|
| 1         | 08.27.2005             | S S S S S R R S S                | -          | -         | A I             |
| 2         | 09.30.2005             | S S S S S R R S S                | +          | +         | A I             |
| 3         | 10.13.2005             | S S S S S R R S S                | +          | -         | A I             |
| 4         | 09.11.2005             | S S S S S R R S S                | +          | -         | A I             |
| 5         | 09.08.2004             | S S S S S R R S S                | +          | A         | VI              |

* ESBL-producing isolates: Isolate 10, 12 had *bla*-CTX-M-15; isolate 11, 35 had both *bla*-CTX-M-15 and *bla*-TEM-1; isolate 24 had both *bla*-TEM-1 and *bla*-SHV-12.
is the first report of the presence bla$\text{SHV-12}$ β-lactamase gene in a $S.\ sonnei$ strain isolated in Turkey.

Nine different plasmid profiles (I-IX) were detected. The size of the plasmids ranged from 2 to 10 kb. Two plasmids with approximately 3- and 5-kb in size were present in more than 90% of the isolates. Twenty-five of the 43 $S.\ sonnei$ isolates were classified in plasmid profile I (Table 1). Plasmid profiles of four bla$\text{CTX-M-15}$-producing isolates were the same (profile I). Although it was indicated that plasmid profile analysis is one of the appropriate methods for molecular characterization of $Shigella$ isolates recovered over short time periods (12), we could not find any epidemiological link among the isolates classified in the same plasmid profile. For instance, the predominant plasmid profile I included more than half of the isolates collected from 2004 to 2006.

PFGE typing of the 43 $S.\ sonnei$ isolates yielded 10 different profiles, six (A, B, C, D, E, and F) were indistinguishable profiles including 23, 3, 4, 3, 2, and 4 isolates, respectively and the remaining four (A1, C1, D1, and E1) were closely related profiles having 1 to 2 band-differences from their related clones. Genetic heterogeneity among different PFGE profiles was very low. According to similarity percentage of $>82\%$, two major PFGE profiles were determined, Group 1 included 39 isolates, and Group 2 included four isolates. There were 1-2 band-differences among PFGE profiles in major group I, while there were four differences between major group 1 and group 2 (Figure 1).

We observed limited correlation between the typing results and epidemiological characteristics. Plasmid profile I was found in five distinct PFGE types (PFGE types A, B, C, D, and F) and a common PFGE type (PFGE type A) were found in six different plasmid profiles (profile I, II, V, VI, VII, and IX). In parallel to our data, Pichel et al. (2007) found similar plasmid profiles in distinct PFGE clones and five different plasmid profiles in one outbreak clone. Four $\text{CTX-M-15}$-producing isolates showed three different PFGE types, two plasmid profiles, and two antibiotypes. PFGE type A was predominant both in phase 1 and phase 1+2 serotypes. Twenty-three (82%) of the 28 isolates classified in the phase 1 serotype, 5 (62%) of the 8 isolates classified in the phase 2, and 6 (86%) of the 7 isolates classified in the phase 1+2 showed the type 2 antibiotic resistance phenotype. There were no correlation between PFGE types and antibiogram types, however at least half of the isolates of each antibiotic type showed PFGE profile A. Although the previous studies revealed that PFGE was the most discriminatory method among subtyping methods (DeLappe et al., 2003; Na-Ubol et al., 2006; Pichel et al., 2007), the $S.\ sonnei$ isolates analyzed in the current study showed very low genetic diversity and there was no direct epidemiological relationship among the isolates having indistinguishable PFGE profiles. For instance, 23 isolates classified in PFGE profile “A” were isolated from the first week of September 2004 to the last week of September 2006. On the other hand, the strains isolated from patient 2 and patient 7 with an interval of three days showed different PFGE profiles “E1” and “C”, respectively. In parallel to our results, a study carried on the $Shigella$ isolates from Ankara has also indicated high relationship among the tested isolates (Saran et al., 2013). These findings support the comment that PFGE is a powerful tool for shigellosis outbreak investigation. However it has some disadvantages for the investigation of clonal relationships among $S.\ sonnei$ strains circulating over a period of months or years (Liang et al., 2007).

In conclusion, this is the first report indicating the presence of bla$\text{SHV-12}$ in $S.\ sonnei$ in Turkey. High genetic homology among the sporadic $S.\ sonnei$ isolates may indicate ongoing cross-transmission for a long period rather than a recent transmission.
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