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Antimicrobial Resistance Patterns and Characterization of Integrons of *Shigella sonnei* Isolates in Seoul, 1999-2008

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A total of 66 *Shigella sonnei* isolates from 1999 to 2008 in Seoul was analyzed for their antimicrobial resistance, carriage of integron, and the patterns of Pulsed-field gel electrophoresis (PFGE). A high level of antimicrobial resistance to streptomycin (100%), trimethoprim/sulfamethoxazole (95%), tetracycline (94%), nalidixic acid (65%), and ampicillin (41%) was observed among *S. sonnei* isolates. Fourteen profiles of antimicrobial resistance were identified with the most common resistance profile being nalidixic acid, streptomycin, tetracycline, and trimethoprim/sulfamethoxazole (35%). PCR and DNA sequencing analysis revealed the presence of class 2 integron in all isolates, and class 1 and 2 integrons in 7 isolates. The class 2 integron carried two types of gene cassettes. One cassette array was *dfrI*, *sat2*, and *aadA1* (91%), and the other was *dfr1* and *sat1* (8%). *dfrA12* and *aadA2* gene cassette was found in one isolate containing class 1 integron. PFGE was carried out to examine the genetic relatedness among isolates. All isolates except for one showed similar PFGE patterns (similarity of 80.1%). These results suggest that the *S. sonnei* isolated during 1999-2008 in Seoul have similar lineages that have not undergone evolutionary changes with time.

***Keywords*:** *S. sonnei*, antimicrobial resistance, integron, PFGE

Shigellosis is an acute diarrheal disease caused by bacteria of the genus *Shigella*, and is a major public health problem in both developing and industrialized countries (Niyogi, 2005). Since 1998 there has been a steady increase in the incidence of shigellosis caused by *Shigella sonnei* in Korea (Lee *et al*., 2006). The annual incidence of shigellosis was estimated to be 2,462 cases in 2000, which then decreased gradually to 131 cases in 2008.

Antimicrobial therapy reduces the duration of clinical symptoms and fecal excretion of the organism. The selection of specific antimicrobial agents should be made based on the susceptibility of the organism or information on local susceptibility patterns. Third generation cephalosporins and quinolones are the mainstay of treatment because *Shigella* isolates have been reported to show resistance to first-line antibiotics (Lee *et al*., 2006). Quinolones, such as norfloxacin or ciprofloxacin, are one of the few remaining groups of effective drugs (Oh *et al*., 2003). Recently, however, a few cases of ciprofloxacin- and other fluoroquinolones-resistant *Shigella* spp. have been reported. (Bhattacharya *et al*., 2003; Naheed *et al*., 2004). Resistance dissemination among *Shigella* spp. is facilitated by the ability of this genus to acquire mobile genetic elements, such as plasmids or transposons. Moreover, these elements may harbor integrons that can integrate resistance gene cassettes by site-specific recombination and provide an efficient means for determining the cumulating resistance (Hall and Collis, 1995; Ploy *et al*., 2000).

Many resistance genes, such as *dfr* genes, *aad* genes, and β-lactamase genes, present as gene cassettes in an integron. Hence, these resistance genes can move to other genetic sites or transfer horizontally to other bacteria (Hall and Collis, 1995). Such genetic transfer is of particular significance when the new host is a pathogenic organism and the inserted gene confers resistance to a widely used antimicrobial agent (Maguire *et al*., 2001).

Pulsed-field gel electrophoresis (PFGE) is a broadly applicable typing method with a high degree of intra- and inter-laboratory reproducibility when standardized protocols are followed (Centers for Disease Control and Prevention, 2000). PFGE was proved to be a powerful tool in the laboratory for discriminating *Shigella* strains during an outbreak (Chiou *et al*., 2001). For these reasons, PFGE was used as a primary typing technique in this study. An understanding of the antibiotic resistance patterns of *S. sonnei* and molecular characterization of the other genetic elements are also epidemiologically useful. In particular, the patterns of antibiotic resistance, which vary according to their location, are in a continuous state of evolution and must also be updated (Niyogi, 2005).

Research on the antimicrobial resistance pattern of *S. sonnei* in 2004 was reported by several authors in Korea, but there are few reports on the latest strains. This study examined the changes in the patterns of antimicrobial resistance, integron carriage, and PFGE patterns of *S. sonnei* isolated in Seoul, Korea during 1999-2008.

Materials and Methods

Bacterial isolates

From 1999 to 2008, a total of 358 *S. sonnei* strains were isolated from

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diarrhea patients at the Seoul Metropolitan Government Research Institute of Public Health and Environment (SIHE) in Seoul, Korea. The following isolates were used in this study: 41 randomly chosen isolates out of 307 isolates identified in the years 1999-2002; 9 randomly chosen isolates out of 35 isolates identified in 2005; 16 isolates identified in 2003, 2004, 2006, 2007, and 2008. A total of 66 isolates was selected for this study. All strains were identified at the genus and species levels using the API 20E system (bioMerieux, France) and antisera (Denka Seiken, Japan). The isolates were stored at -70°C.

Antibiotic susceptibility test

Antibiotic susceptibility of these isolates was determined using a disc diffusion method, according to the guidelines of the Clinical Laboratories Standards Institute (CLSI). Twenty antimicrobial agents (Becton and Dickinson, USA) were used. Ampicillin (AM), ampicillin/sulbatam (SAM), cephalothin (CF), streptomycin (S), kanamycin (K), ceftriaxone (CRO), ciprofloxacin (CIP), chloramphenicol (C), gentamicin (GM), nalidixic acid (NA), tetracycline (TE), and trimethoprim/sulfamethoxazole (SXT) are among them. *Escherichia coli* ATCC 25922 was used as a quality control strain.

DNA sequence analysis of the quinolone resistance-determining region (QRDR)

This study searched for changes in the QRDRs nucleotide sequence of 21 isolates that showed low susceptibility to ciprofloxacin and resistance to nalidixic acid. The *gyrA* gene for DNA gyrase and *par*C gene for topoisomerase IV in 21 *S. sonnei* isolates were amplified by PCR (Giraud *et al*., 1999), and sequenced using an ABI 3700 sequencer (Applied Biosystems, USA). These portions of the QRDR corresponded to the amino acid residues 54-171 of GyrA, and 12-130 of ParC.

Integron analysis

DNA was extracted from the bacteria using a boiling method, and the integrons were detected by PCR with the primers hep35 (5′-TGCGG GTYAARGATBTKGATTT-3′) and hep36 (5′-CARCACATGCGTRT ARAT-3′), which hybridize to the conserved regions of integron-encoded integrase genes *intI1*, *intI2*, and *intI3* (White *et al*., 2000). The class of the integrons was determined by analyzing the PCR products with restriction fragment length polymorphism (RFLP) after digestion using a restriction enzyme, *Hinf*I. The gene cassette regions for the class 1 and 2 integrons were amplified using the primer pairs, hep58 (5′-TCATGGCTTGTTATGACTGT-3′) and hep59 (5′-GTAGGGCTT ATTATGCACGC-3′), and hep74 (5′-CGGGATCCCGGACGGCATG CACGATTTGTA-3′) and hep51 (5′-GATGCCATCGCAAGTACGAG-3′), respectively, as described previously (White *et al*., 2000, 2001). The amplified DNA products of interest were gel extracted and purified (QIAGEN, UK), and then sequenced using automated methods. The resulting DNA sequence was analyzed using the software available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov.).

Pulsed field gel electrophoresis (PFGE)

PFGE was performed using the standardized PulseNet protocol (Centers for Disease Control and Prevention, 2004). PFGE plugs were digested with the restriction enzyme *Xba*I and separated in a 1% agarose gel with a CHEF Mapper PFGE system (BioRad, USA). Analysis of the PFGE banding patterns was performed using Bionumerics software (Applied Maths, Belgium). The banding patterns were analyzed with the Dice coefficient using 1.5% tolerance for the band migration distance.

Results

Antibiotic resistance patterns

Table 1 summarizes the antimicrobial resistance phenotypes of the isolates. The *S. sonnei* isolates were all resistant to streptomycin (S), 95% of them were resistant to sulfamethoxazole/trimethoprim (SXT), 94% were resistant to tetracycline (TE), 65% were resistant to nalidixic acid (NA), 41% were resistant to ampicillin (AM), 26% were resistant to kanamycin (K), and 9% were resistant to gentamycin (GM). No isolate was resistant to chloramphenicol (C) and ciprofloxacin (CIP).

Multidrug resistance to the 14 groups was also observed in the *S. sonnei* isolates (Table 2). The most frequent multiple resistance was to 4 antimicrobials, including NA, S, TE, and SXT (35%). Seven isolates (12%) were resistant to 5 antimicrobials, including AM, K, S, TE, and SXT. All outbreak-related isolates (12 isolates) from 2001 except for 3 isolates were resistant to NA, S, TE, and SXT. Among the 3 isolates

Table 1. Resistance of the isolated *S. sonnei* strains to antimicrobial drugs

Year	No. of isolates	No.(%) of isolates resistant to											
		AM	K	GM	SAM	CF	CRO	NA	CIP	C	S	TE	SXT
1999	13	6(46)	4(31)	0	1(8)	1(8)	0	13(100)	0	0	13(100)	12(92)	12(92)
2000	10	6(60)	4(40)	0	1(10)	0	0	5(50)	0	0	10(100)	9(90)	9(90)
2001	15	3(20)	3(20)	0	1(7)	1(7)	0	12(80)	0	0	15(100)	15(100)	15(100)
2002	3	1(33)	3(100)	0	0	0	0	2(67)	0	0	3(100)	3(100)	3(100)
2003	5	3(60)	3(60)	0	0	0	0	1(20)	0	0	5(100)	5(100)	5(100)
2004	2	0	0	0	0	0	0	0	0	0	2(100)	2(100)	2(100)
2005	9	6(67)	0	5(56)	0	1(11)	1(11)	6(67)	0	0	9(100)	8(89)	8(89)
2006	3	2(67)	0	1(33)	0	0	0	2(67)	0	0	3(100)	3(100)	3(100)
2007	3	0	0	0	0	0	0	2(67)	0	0	3(100)	2(67)	3(100)
2008	3	0	0	0	0	0	0	0	0	0	3(100)	3(100)	3(100)
	66	27(41)	17(26)	6(9)	3(4.5)	3(4.5)	1(1.5)	43(65)	0	0	66(100)	62(94)	63(95)

AM, Ampicillin; K, Kanamycin; GM, Gentamycin; SAM, Ampicillin/Sulbactam; CF, Cephalothin; CRO, Ceftriaxone; NA, Nalidixic acid; CIP, Ciprofloxacin; C, Chloramphenicol; S, Streptomycin; TE, Tetracycline; SXT, Sulfamethoxazole/Trimethoprim

Table 2. Distribution of the multiple antimicrobial resistance patterns of *S. sonnei* isolates, 1999-2008

Group	Resistance pattern	No. of strains isolated in										No. of isolates
		1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	
R1	NA-S	1										1
R2	S-SXT									1		1
R3	S-TE-SXT		2			1	2	3	1		3	12
R4	NA-S-TE-SXT	6	2	12[a]		1				2		23
R5	AM-K-S-SAM		1									1
R6	AM-NA-S-TE-SXT	2	2						1			5
R7	AM-K-S-TE-SXT		2	1	1[b]	3						7
R8	K-NA-S-TE-SXT				2[b]							2
R9	AM-CF-CRO-NA-S							1				1
R10	AM-K-NA-S-TE-SXT	3	1									4
R11	AM-K-SAM-S-TE-SXT			1								1
R12	AM-K-CF-S-TE-SXT			1								1
R13	AM-GM-NA-S-TE-SXT							5[c]	1			6
R14	AM-K-S-SAM-CF-NA-TE-SXT	1										1
	Total	13	10	15	3	5	2	9	3	3	3	66

AM, Ampicillin; K, Kanamycin; GM, Gentamycin; SAM, Ampicillin/Sulbactam; CF, Cephalothin; CRO, Ceftriaxone; NA, Nalidixic acid; CIP, Ciprofloxacin; C, Chloramphenicol; S, Streptomycin; TE, Tetracycline; SXT, Sulfamethoxazole/Trimethoprim

[a] Twelve strains were isolated from the outbreak in 2001

[b] Three strains were isolated from the outbreak in 2002

[c] Five strains were isolated from the outbreak in 2005

isolated during the outbreak in 2002, one was resistant to AM but the other two were susceptible. On the other hand, these two were resistant to nalidixic acid. All isolates from 2003 were sporadic cases. Five outbreak-related isolates from 2005 were resistant to 6 antimicrobials including GM. One isolate from 1999 was resistant to 8 antibiotics (AM, K, S, SAM, CF, NA, TE, SXT).

DNA sequence analysis of the quinolone resistance-determining region (QRDR)

Twenty-one isolates that showed low susceptibility to ciprofloxacin and resistance to nalidixic acid were searched for QRDRs nucleotide sequence changes. All the isolates showed a mutation in *gyrA* but no mutations in the *parC* gene. One mutation found in 21 of the isolates was located at codon 83 of *gyrA* (TCG→TTG transition), resulting in the replacement of serine with leucine (data not shown).

Integron analysis

All isolates were found to harbor integrons of one or two classes: 66 strains possessed a class 2 integron (100%), and 7 strains carried both class 1 and 2 integrons. No class 3 integrons were detected. Two different types of cassette arrays were detected in the class 2 integrons. Sixty isolates contained a 2,158 bp gene cassette identified by DNA sequence analysis as containing *dhfrI*, *sat2*, and *aadA1* (GenBank accession no. AB234885). Five isolates contained a smaller cassette structure of 1,456 bp encoding only two genes within the cassette. The gene cassette showed 100% identity with *dhfr1*, *sat1* (GenBank accession no. AM745943). Seven isolates also had a class 1 integron but no cassette regions were found in the 6 isolates. Only one class 1 integron harbored the *dfrA12* and *aadA2* cassette array (GenBank accession no. GQ280258). Table 3 shows the characteristics of *S. sonnei* used in this study.

PFGE analysis

Five major clusters of *S. sonnei* were identified by PFGE (Fig. 1): PFGE type A (n=46), type B (n=11), type C (n=4), type D (n=4), and type E (n=1). The similarity indexes for each of the above groups were 90.2%, 91.8%, 89.6%, and 95.2%, respectively. The similarity index for all groups was 70.5%. The largest group, type A appeared to be quite homogeneous with 6 subgroups detected (A1-A6); Type B was isolated in 2000, 2001, 2003, and 2005, and it was similar to A by 87.7%; Type C was formed with two isolates, one isolated in 2003 and the other isolated in 2008 ; Type D consisted of *dhfr1-sat1* cassette carriage isolates ; and Type E was slightly different from other 65 *S. sonnei* isolates. Type E strain was isolated from woman who came back from Philippines trip in 2007. This strain was resistant to streptomycin and trimethoprim/sulfamethoxazole and the cassette of class 2 integron was not found.

Discussion

There are reports of *Shigella* spp. isolates with the streptomycin, sulfonamide, tetracycline, and trimethoprim resistance phenotype from a number of countries, such as Australia (Mclver *et al*., 2002), Brazil (Peirano *et al*., 2005), Japan (Ahmed *et al*., 2006), and Italy (Mammina *et al*., 2005). Most of *S. sonnei*, isolated for 10 years in Seoul, were resistant to streptomycin, tetracycline, and trimethoprim/sulfamethoxazole. In particular, the *S. sonnei* in this study were resistant to nalidixic acid (65%). This is similar to the antibiotic resistance pattern before 2004 in Korea (Oh *et al*., 2003; Huh *et al*., 2007). Resistance to quinolones is usually due to the result of

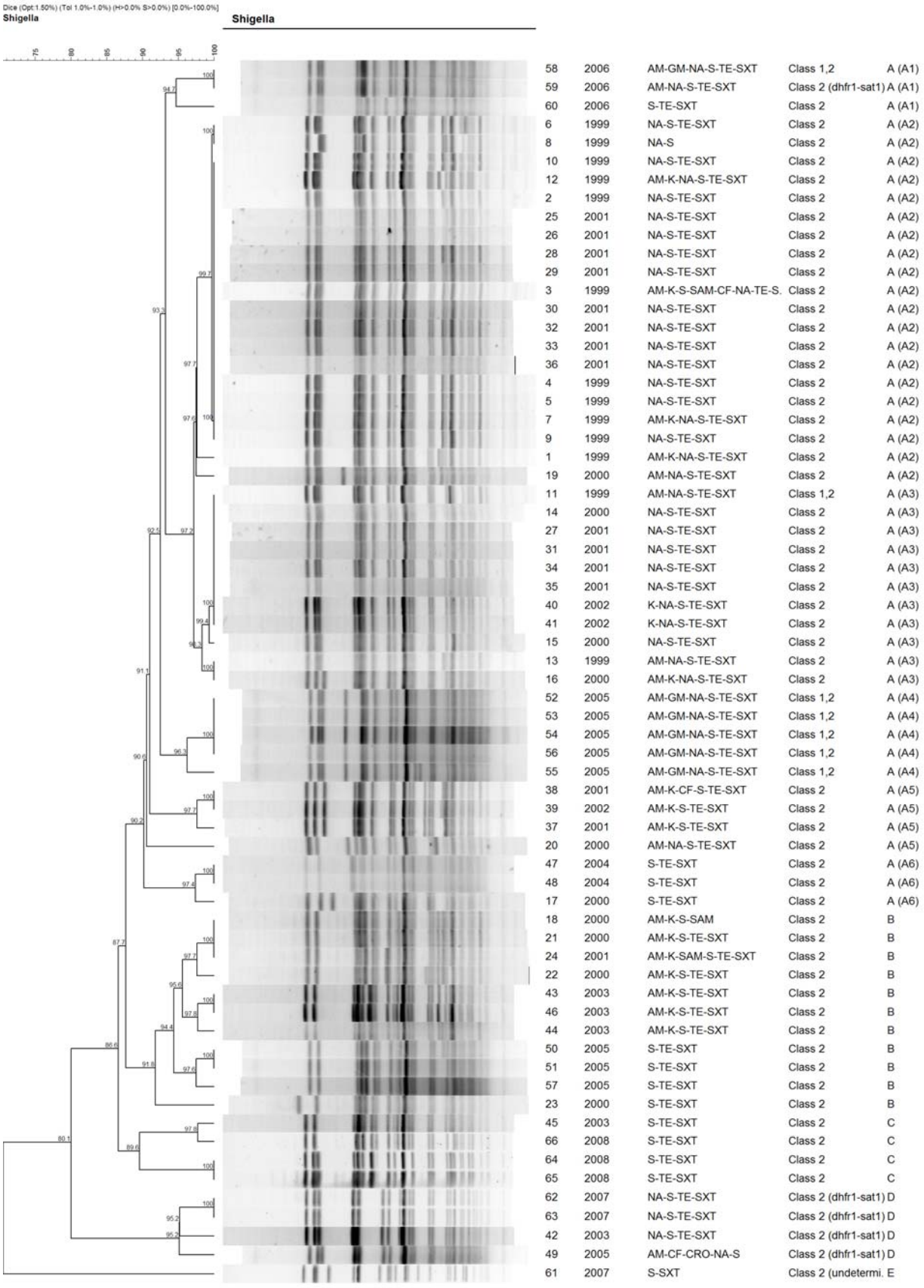

Fig. 1. Dendrongram showing the clustering of PFGE patterns after *Xba*I digestion for the *S. sonnei* isolates.

Table 3. Characteristic features of *S. sonnei* used in this study

No.	Year of isolation	Resistance phenotype	No. of isolates	Integron
1	1999	NA-S	1	Class 2(*dfrI*, *sat2*, *aadA*)
2		NA-S-TE-SXT	6	Class 2(*dfrI*, *sat2*, *aadA*)
3		AM-K-NA-S-TE-SXT	3	Class 2(*dfrI*, *sat2*, *aadA*)
4		AM-NA-S-TE-SXT	2[a]	Class 1(*dfrA12*, *aadA2*)+Class 2(*dfrI, sat2, aadA*) Class 2(*dfrI*, *sat2*, *aadA*)
5		AM-K-S-SAM-CF-NA-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
6	2000	S-TE-SXT	2	Class 2(*dfrI*, *sat2*, *aadA*)
7		NA-S-TE-SXT	2	Class 2(*dfrI*, *sat2*, *aadA*)
8		AM-K-S-SAM	1	Class 2(*dfrI*, *sat2*, *aadA*)
9		AM-NA-S-TE-SXT	2	Class 2(*dfrI*, *sat2*, *aadA*)
10		AM-K-S-TE-SXT	2	Class 2(*dfrI*, *sat2*, *aadA*)
11		AM-K-NA-S-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
12	2001	NA-S-TE-SXT	12	Class 2(*dfrI*, *sat2*, *aadA*)
13		AM-K-S-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
14		AM-K-CF-S-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
15		AM-K-SAM-S-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
16	2002	AM-K-S-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
17		K-NA-S-TE-SXT	2	Class 2(*dfrI*, *sat2*, *aadA*)
18	2003	S-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
19		NA-S-TE-SXT	1	Class 2(*dfr1*, *sat1*)
20		AM-K-S-TE-SXT	3	Class 2(*dfrI*, *sat2*, *aadA*)
21	2004	S-TE-SXT	2	Class 2(*dfrI*, *sat2*, *aadA*)
22	2005	S-TE-SXT	3	Class 2(*dfrI*, *sat2*, *aadA*)
23		AM-CF-CRO-NA-S	1	Class 2(*dfr1*, *sat1*)
24		AM-GM-NA-S-TE-SXT	5	Class 1(undetermined)+Class 2(*dfrI*, *sat2*, *aadA*)
25	2006	S-TE-SXT	1	Class 2(*dfrI*, *sat2*, *aadA*)
26		AM-NA-S-TE-SXT	1	Class 2(*dfr1*, *sat1*)
27		AM-GM-NA-S-TE-SXT	1	Class 1(undetermined)+Class 2(*dfrI*, *sat2*, *aadA*)
28	2007	S-SXT	1	Class 2 (undetermined)
29		NA-S-TE-SXT	2	Class 2(*dfr1*, *sat1*)
30	2008	S-TE-SXT	3	Class 2(*dfrI*, *sat2*, *aadA*)

AM, Ampicillin; K, Kanamycin; GM, Gentamycin; SAM, Ampicillin/Sulbactam; CF, Cephalothin; CRO, Ceftriaxone; NA, Nalidixic acid; CIP, Ciprofloxacin; C, Chloramphenicol; S, Streptomycin; TE, Tetracycline; SXT, Sulfamethoxazole/Trimethoprim

[a] One strain was harbored class 1, 2 integrons

mutations in the bacterial targets, DNA gyrase and topoisomerase IV, or due to the activation of efflux pumps (Kim *et al*., 2008). Twenty-one clinical isolates with reduced susceptibility to fluoroquinolones were found to contain mutations in *gyrA*. The mutation at the codon specifying Serine 83 in GyrA, which produced a change to leucine, was observed in all 21 isolates. The mutation found in this study has been reported previously (Seol *et al*., 2005; Hu *et al*., 2007; Kim *et al*., 2008) Besides *S. sonnei*, it was reported that a substitution of codon 83-serine for leucine in quinolone resistant *E. coli* is most prevalent (Saenz *et al*., 2003). Quinolone-resistant *S. sonnei* isolates obtained over a 10 year period in Seoul did not undergo the evolutionary changes with the time in QRDRs. In early 2000, *S. sonnei* isolates that were resistant to ampicillin and kanamycin had changed to susceptible strains, which was associated with selective pressure of antibiotics and highlights the need to monitor newly introduced antibiotics.

Of the three classes of integrons associated with antimicrobial resistance, class 1 integron is the most commonly found in clinical isolates of Gram-negative bacteria (Goldstein *et al*., 2001). Class 2 integron, however, was the most predominant integron in *S. sonnei* (Hansson *et al*., 2002.) All 66 strains in this study had the class 2 integron and 7 isolates also contained class 1 integron. PCR and DNA sequencing results identified two types of class 2 integron. One was the classic type and carried a gene cassette array analogous to that found in Tn7 (*dfrA*1, *sat1*, and *aadA*1), which conferred resistance to trimethoprim, streptothricin, and spectinomycin/streptomycin, respectively (Mclver *et al*., 2002). The other type was shorter and carried *dfr1-sat1* gene cassettes that encoded resistance to trimethoprim and streptothricin. All cassette arrays of the class 2 integrons found in this study were detected previously in *S. sonnei* isolated from other studies (Ahmed *et al*., 2006; Mammina *et al*., 2006; Ranjbar *et al*., 2007). In general, class 2

integron's cassette array of *S. sonnei* is relatively regular. This could be explained by the fact that the class 2 integrase gene (*IntI2*) contains an early stop codon resulting in a truncated form of the enzyme. The resultant integrase is therefore unable to excise existing cassettes or insert new ones (White *et al*., 2001). Among the 66 *S. sonnei* strains analyzed, only one class 1 integron isolate (isolate from 1999) contained *dfrA12-aadA2* gene cassette that encoded resistance to trimethoprim and spectinomycin/streptomycin. This integron was detected in *S. sonnei* that was isolated in the southwestern Korea during epidemic periods (Oh *et al*., 2003). In addition, the same cassette has been found in different *Enterobacteriaceae* (Lee *et al*., 2001). Although antibiotics, such as streptomycin and spectinomycin, cannot be used therapeutically, genes encoding resistance to these antibiotics are not erasable, so this type of genes are re-exposed and expressed efficiently (Hall and Collis, 1995). This study did not identify a gene cassette conferring resistance to recently introduced antibiotics. These cassettes have not yet received sufficient selective pressure over the evolutionary timescale to encourage their widespread dissemination. However, continuous monitoring is important.

This study was intended to provide baseline data for the application of PFGE to the routine typing of *S. sonnei* isolates as well as to examine the change in genetic pattern of *S. sonnei* in Seoul, Korea over one decade. The correlation of the PFGE type with the results of phenotypic typing methods is useful for epidemiological purposes. According to Lee's study (Lee *et al*., 2006), the characteristics of the Korean epidemic *S. sonnei* clone from 1998 to 2004 were defined by biotype g, arbitrarily PFGE pattern II, and the common resistant to tetracycline, streptomycin, sulfonamide, and trimethoprim. Similarly, *S. sonnei* isolated from Australia, Italy, Ireland, USA, and African countries during the same period exhibited biotype g and similar PFGE patterns to the Korean *S. sonnei* isolates (Mclver *et al*., 2002; Mammina *et al*., 2005), suggesting the existence of a pandemic *S. sonnei* clone. In this study, with the exception of one isolate, most of the isolates were similar to each other, showing 80.1% homology. One isolate isolated in 2007 showed unique PFGE pattern, a different antibiotic resistance pattern and integron condition. This stain is not domestic endemic clone and it came from foreign. It is identified that PFGE is useful as an epidemiological tool. Overall, it is essential to prevent the spread of antibiotic resistance and diffusion of integrons should be prevented through continuous monitoring.

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