Molecular characterization of antimicrobial resistance in *Shigella sonnei* isolates in Korea

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The antimicrobial resistance of 122 *Shigella sonnei* isolates obtained in Korea during the period 1991–2000 was characterized. These isolates were highly resistant to traditional antibiotics such as trimethoprim (100%), streptomycin (100%), sulfamethoxazole (94%), tetracycline (93%) and nalidixic acid (90%). All *S. sonnei* isolates carried Tn7 in their chromosomes. The 8.4 kb non-transferable resistance (R) plasmid carrying *tetA*, *strA-strB* and *sul1* was found in 93% of the *S. sonnei* isolates. Resistance to nalidixic acid first appeared in a *S. sonnei* isolate in 1997, and then in all *S. sonnei* isolates from 1998 and 1999. Resistance to commonly prescribed antibiotics such as ampicillin was increased in *S. sonnei* isolates during the outbreak period 1998–2000. Resistance to ampicillin was mediated by the conjugative R plasmids carrying *bla*_{TEM-1}. In conclusion, *S. sonnei* acquired antimicrobial resistance to commonly prescribed antibiotics through the horizontal transfer of conjugative R plasmids, while the genetic stability of transposon and non-transferable R plasmids was responsible for resistance to traditional antibiotics.

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INTRODUCTION

Shigellosis remains an important cause of community-acquired gastrointestinal illness in both developing and industrialized countries. Since 1951, when *Shigella sonnei* was first reported to be sporadically isolated in Korea (Im & Choi, 1961), *S. sonnei* has become, in the 1990s and 2000s, the primary cause of shigellosis (Kim *et al.*, 2002; Oh *et al.*, 2003). Shigellosis caused by *S. sonnei* occurred sporadically in Korea until 1997, when the annual incidence was estimated to be 2–260 cases, but large outbreaks of shigellosis caused by *S. sonnei* have occurred since 1998, and the annual incidence of *S. sonnei* is now estimated to be 846–2400 cases (National Institute of Health in Korea, 2005). We previously reported that there are distinct phenotypic and genotypic differences in terms of biotypes, antimicrobial susceptibilities and PFGE profiles between the *S. sonnei* isolates from the 1980s and the 1990s (Jeong *et al.*, 2003; Kim *et al.*, 2002; Oh *et al.*, 2003). *S. sonnei* isolates during the period 1977–1986 exhibited biotype a, while *S. sonnei* isolates during the period 1991–2000 exhibited biotype g. PFGE patterns of *S. sonnei* isolates during the period 1977–1986 were completely different from those of isolates during the period 1991–2000. This suggests that Korean endemic *S. sonnei* strains disappeared, and that new

S. sonnei clones were introduced in the late 1980s or early 1990s.

In Korea, *S. sonnei* isolated during the last decade were resistant to multiple antibiotics, and a high proportion of *S. sonnei* were commonly resistant to tetracycline, streptomycin, sulfonamide and trimethoprim (Jeong *et al.*, 2003). Additionally, antimicrobial susceptibilities of *S. sonnei* isolates gradually changed over time. The most striking changes were an increase in resistance to ampicillin and the emergence of extended-spectrum β -lactamases (ESBLs) in *S. sonnei* isolates. ESBLs, including TEM-15, TEM-17, TEM-19, TEM-20, TEM-52 and CTX-M-14, have been detected among recently identified *S. sonnei* isolates (Kim *et al.*, 2004; Pai *et al.*, 2001). However, there have been no comprehensive studies involving the molecular characterization of antimicrobial resistance in the *S. sonnei* isolates in Korea. The current study investigated the molecular mechanisms by which *S. sonnei* isolates during the period 1991–2000 were resistant to antimicrobial agents.

METHODS

Bacterial isolates. A total of 122 *S. sonnei* isolates were obtained from stool samples in different parts of Korea during the period 1991–2000 (Table 1). All *S. sonnei* isolates from sporadic shigellosis were collected. From the defined outbreaks, *S. sonnei* isolates that showed the same antibiogram and identical or similar PFGE pattern (similarity value of >95%) were considered to be genetically

Abbreviations: ESBL, extended-spectrum β -lactamase; R plasmid, resistance plasmid.

Table 1. Transferability of antimicrobial resistance in *S. sonnei* isolates

Abbreviations: Tc, tetracycline; Sm, streptomycin; Su, sulfamethoxazole; Ap, ampicillin; Tp, trimethoprim; Km, kanamycin; Na, nalidixic acid; Cm, chloramphenicol; Gm, gentamicin; Cp, cefoperazone. Parentheses indicate number of strains.

Year isolated	Isolation area	No. of isolates	Antimicrobial resistance	No. of trans-conjugants	Transferable anti-microbial resistance
1991	Unknown (6)	5	TcSmSuApTpKm	5	SmSuApTpKm (1), ApKm (4)
		1	SmSuApTpKm	1	SmSuApTpKm (1)
1992–1995	Unknown (3)/Seoul (1)/ Kyungpook (1)	5	TcSmSuTp	0	—
1997	Kyungpook (1)	1	TcSmSuTpNa	0	—
1998	Kyungpook (28)	28	TcSmSuTpNa	0	—
	Kyungpook (1)	1	TcSmSuApTpNa	1	Ap (1)
	Kyungpook (1)	1	SmTpNa	0	—
1999	Kyungpook (28)/Chunnam (27)	55	TcSmSuTpNa	0	—
	Kyungpook (6)	6	SmTpNa	0	—
	Kyungpook (3)/Chunnam (2)	5	TcSmSuApTpNa	5	SmSuApTp (3), TcSmSuAp (1), TcSmSu (1)
	Chunnam (4)	4	TcSmSuApTpKmNa	4	SmSuApKm (1), ApKm (3)
	Chunnam (3)	3	CmTcSmSuTpNa	3	CmSmSu (3)
	Kyungpook (1)	1	TcSmSuApCpTpGmNa	1	SmSuApCpTpGm (1)
	Kyungpook (1)	1	TcSmSuTpKmGmNa	1	SmSuTpKmGm (1)
2000	Kyungpook (2)/Chunnam (1)	3	TcSmSuTpNa	0	—
	Chunnam (1)	1	TcSmSuApTpKm	1	ApKm (1)
	Kyungpook (1)	1	TcSmSuApTpKmNa	1	TcSmSuApKm (1)

identical or closely related (Tenover *et al.*, 1995), and a representative isolate was collected. Isolates were identified to genus and species level by conventional biochemical and serological methods (Ewing, 1986). Serological identification was performed by tube and slide agglutination with antisera (Difco Laboratories).

Antimicrobial susceptibility testing. The MICs of the antimicrobial agents were determined by the agar dilution method in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (1997). The inoculated plates were incubated at 37 °C for 20 h. The MIC was defined as the lowest concentration of an antimicrobial agent that completely inhibited the growth of organisms. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains for quality control. Antimicrobial resistance was determined according to the National Committee for Clinical Laboratory Standards breakpoints, except for streptomycin, as follows: ampicillin (≥ 32 mg l⁻¹), cefoperazone (≥ 64 mg l⁻¹), streptomycin (≥ 32 mg l⁻¹) (Voogd *et al.*, 1992), kanamycin (≥ 64 mg l⁻¹), gentamicin (≥ 16 mg l⁻¹), nalidixic acid (≥ 32 mg l⁻¹), ciprofloxacin (≥ 4 mg l⁻¹), chloramphenicol (≥ 32 mg l⁻¹), sulfamethoxazole (≥ 512 mg l⁻¹), trimethoprim (≥ 16 mg l⁻¹) and tetracycline (≥ 16 mg l⁻¹).

Conjugal transfer of antimicrobial resistance, plasmid analysis and Southern hybridization. All *S. sonnei* isolates were included as putative donors in a conjugation experiment. *E. coli* RG488 Rif^r and J53 Azi^r were used as recipients to detect the transfer of resistance (R) plasmids (Kim *et al.*, 2002). Donor and recipient strains at exponential phase were grown in Trypticase soy broth (Difco Laboratories) and were mixed and incubated at 37 °C for 20 h. Transconjugants were selected on Mueller–Hinton agar (Difco Laboratories) supplemented with the combination of rifampicin (50 µg ml⁻¹) or sodium azide (50 µg ml⁻¹), and with tetracycline (20 µg ml⁻¹), streptomycin (32 µg ml⁻¹), trimethoprim (50 µg ml⁻¹) or ampicillin (50 µg ml⁻¹). Plasmid DNAs from clinical

isolates or their transconjugants were isolated by the alkaline extraction method (Birnboim & Doly, 1979). Plasmid DNAs were digested with *EcoRI* or *SmaI* restriction enzymes (Boehringer Mannheim) and separated by electrophoresis in 0.8% agarose gels. For Southern hybridization, the denatured DNAs were transferred to a positively charged nylon membrane and hybridized with the probes. The probes were labelled with digoxigenin-11-dUTP by random labelling methods (Boehringer Mannheim). A digoxigenin DNA labelling and detection kit was used according to the manufacturer's instructions.

PFGE. Genomic DNA was digested with *XbaI* (Boehringer Mannheim) for 20 h and separated in a 1% agarose gel with a contour-clamped homogeneous electric field apparatus (CHEF-DR III, Bio-Rad). The conditions for electrophoresis were 6 V cm⁻¹ for 21 h, with the pulse time increasing from 5 to 40 s (Kim *et al.*, 2002).

PCR amplification of integrons and Tn3. To determine the presence of class 1 and class 2 integrons, PCR amplification of *intI1* and *intI2* was performed, respectively, as described previously (Oh *et al.*, 2002). The gene cassette regions of the class 1 and class 2 integrons were amplified using primer pairs hep58/hep59 and hep74/hep51, respectively (White *et al.*, 2000, 2001). To determine identical arrays of gene cassettes, same-sized amplicons were digested with *RsaI* and *HinfI* (Boehringer Mannheim). Every PCR product of cassette regions that showed different restriction patterns was ligated with a pGEM-T Easy vector (Promega) and transformed to *E. coli* DH5α cells. Sequencing reactions were performed with a double-stranded plasmid preparation using dideoxy chain termination with T7 and Sp6 primers. Tn3-specific regions, an internal fragment of *tnpR*, were amplified using the Expand Long Template PCR system (Boehringer Mannheim) with a specific primer (5'-GTCTGACGCT-CAGTGAACG-3') (Jeong *et al.*, 2003).

PCR amplification of antimicrobial resistance genes. PCR was performed to amplify a specific antimicrobial resistance gene for

Fig. 1. PFGE patterns of *Xba*I-digested genomic DNA of *S. sonnei* isolates (A) and Southern blot of gel hybridized with a *dfrA1* gene probe (B). Lanes: M, lambda ladder (Bio-Rad); 1, H002 from 1991; 2, H003 from 1991; 3, H012 from 1995; 4, H021 from 1997; 5, H022 from 1998; 6, H025 from 1998; 7, H043 from 1998; 8, H101 from 1999; 9, H115 from 1999; 10, H265 from 2000; 11, H129 from 1999; 12, H206 from 1999; 13, H152 from 2000, 14, H259 from 2000.

each antimicrobial resistance. Primers and amplification conditions for *bla*_{TEM} (Lee *et al.*, 2001b) and *dfr* (Lee *et al.*, 2001a) were as described previously. Amplification of the specific resistance genes was performed with the following primer sets: *strA*-F (5'-ATG GTG GAC CCT AAA ACT CT-3') and *strB*-R (5'-CGT CTA GGA TCG AGA CAA AG-3') for *strA-strB*, *APH3*'-F (5'-GCA TTT TAT CCG TAC TCC TG-3') and *APH3*'-R (5'-AAC CTA TTA ATT TCC CCT CG-3') for *aph3'*, *tetA*-F (5'-GGC CAG ACG TGA AAC CCA ACA-3') and *tetA*-R (5'-CGC GAA GGC AAG CAG GAT G-3') for *tetA*, *sul1*-F (5'-CTT CGA TGA GAG CGG CGG C-3') and *sul1*-R (5'-CGC AAG GCG GAA ACC CGC GCC-3') for *sul1*, and *sul2*-F (5'-TCA TTT TCG GCA TCG TCA ACA-3') and *sul2*-R (5'-GCG CGA AAC AGA CAG AAG CAC-3') for *sul2*. All PCR amplifications were performed using the following amplification scheme: one cycle

of denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min.

RESULTS

Transferable antimicrobial resistance of *S. sonnei* isolates

S. sonnei isolated during the period 1991–2000 showed very similar PFGE patterns (Fig. 1A), but 10 different antimicrobial resistance patterns were detected (Table 1). To determine whether antimicrobial resistance could be transferred, conjugal transfer of R plasmids was performed. Of the 122 *S. sonnei* isolates tested, 22 (18.0%) isolates transferred their antimicrobial resistance to an *E. coli* strain (Table 1). Twenty-one *S. sonnei* isolates transferred one R plasmid, while one isolate from 1999 (H223) transferred two different R plasmids. Eleven different antimicrobial resistance patterns were obtained among the 23 transconjugants. Resistance to ampicillin, gentamicin and kanamycin was transferred to an *E. coli* recipient, except for one ampicillin-resistant *S. sonnei* isolate from 1999. Resistance to tetracycline, trimethoprim, streptomycin and sulfamethoxazole was transferred to an *E. coli* strain in 2.5% (3/122), 4.1% (5/122), 8.2% (10/122) and 8.2% (10/122) of *S. sonnei* isolates, respectively.

Molecular characterization of conjugative R plasmids

Of the 23 transconjugants that transferred antimicrobial resistance, the conjugative R plasmids were classified into 11 groups by their RFLP patterns, antimicrobial resistance patterns, presence of Tn3, class 1 integrons and antimicrobial resistance genes (Table 2). The transconjugants that transferred antimicrobial resistance carried one large plasmid of 70–80 kb; the RFLP patterns of conjugative R plasmids were different (Fig. 2). To determine whether the

Table 2. Characterization of conjugative R plasmids in *S. sonnei* isolates

For abbreviations, see Table 1.

Plasmid (isolate)	No. of isolates	Antimicrobial resistance	Presence of Tn3	Gene cassettes of class 1 integron	Resistance genes
pKY032 (H003)	2	SmSuApTpKm	+	<i>aadA2-orfF-dfrA12</i>	<i>aadA2</i> , <i>sul1</i> , <i>bla</i> _{TEM-1} , <i>dfrA12</i> , <i>aph3'</i>
pKY251 (H025)	1	Ap	—	—	<i>bla</i> _{TEM-1}
pKY1101 (H110)	1	SmSuApKm	—	—	<i>strA-strB</i> , <i>sul2</i> , <i>bla</i> _{TEM-1} , <i>aph3'</i>
pKY1381 (H138)	3	SmSuApTp	+	<i>aadA2-orfF-dfrA12</i>	<i>strA-strB</i> , <i>aadA2</i> , <i>sul1</i> , <i>bla</i> _{TEM-1} , <i>dfrA12</i>
pKY1402 (H140)	1	SmSuApCpTpGm	+	<i>aadA2-orfF-dfrA12</i>	<i>aadA2</i> , <i>sul1</i> , <i>bla</i> _{TEM-1} , <i>bla</i> _{TEM-52} , <i>dfrA12</i>
pKY1511 (H151)	1	TcSmSuApKm	—	—	<i>tetA</i> , <i>strA-strB</i> , <i>sul2</i> , <i>bla</i> _{TEM-1} , <i>aph3'</i>
pKY2061 (H206)	1	SmSuTpKmGm	—	<i>aadA2-orfF-dfrA12</i>	<i>strA-strB</i> , <i>aadA2</i> , <i>sul1</i> , <i>dfrA12</i> , <i>aph3'</i>
pKY2131 (H213)	1	TcSmSuAp	—	—	<i>tetA</i> , <i>strA-strB</i> , <i>sul2</i> , <i>bla</i> _{TEM-1}
pKY2191 (H219)	3	CmSmSu	—	—	<i>strA-strB</i> , <i>sul2</i>
pKY2231 (H223)	1	TcSmSu	—	<i>aadA1</i>	<i>tetA</i> , <i>strA-strB</i> , <i>aadA</i> , <i>sul1</i>
pKY2654 (H265)	8	ApKm	+	—	<i>bla</i> _{TEM-1} , <i>aph3'</i>

Fig. 2. Restriction endonuclease analysis of R plasmids extracted from transconjugants by *EcoRI* (A) and *SmaI* (B). Lanes: M, lambda DNA/*HindIII* marker; 1, pKY032 from H003; 2, pKY1402 from H140; 3, pKY011 from H001; 4, pKY1101 from H110; 5, pKY1381 from H138; 6, pKY2322 from H232; 7, pKY2131 from H213; 8, pKY062 from H006; 9, pKY1292 from H129; 10, pKY1302 from H130; 11, pKY2654 from H265.

conjugative R plasmids carried class 1 or class 2 integrons, the *intI1* and *intI2* genes were amplified. Of the 11 conjugative R plasmids, five carried class 1 integrons. No class 2

integrons were found in any of the transconjugants. Among the five conjugative R plasmids carrying class 1 integrons, four carried a gene cassette of *dfrA12-orfF-aadA2* and one carried *aadA1*. A class 1 integron carrying *dfrA12-orfF-aadA2* was found in *S. sonnei* isolates from 1991, 1999 and 2000, while a class 1 integron carrying *aadA1* was found in the isolate from 1999.

The mechanisms by which the transconjugants were resistant to antimicrobial agents were determined. Resistance to tetracycline, kanamycin, ampicillin and trimethoprim was mediated by *tetA*, *aph3'*, *bla*_{TEM-1} and *dfrA12*, respectively (Table 2). Resistance to cefoperazone was mediated by *bla*_{TEM-52} in one *S. sonnei* isolate from 1999 (Jeong *et al.*, 2003). Resistance to sulfamethoxazole was mediated by *sul1* in the five class 1 integron-carrying R plasmids, and *sul2* in the four R plasmids that did not contain class 1 integrons. Resistance to streptomycin was mediated by *strA-strB*, *aadA2* or *aadA1*.

Molecular characterization of non-transferable antimicrobial resistance

To characterize the non-transferable antimicrobial resistance among *S. sonnei* isolates, PCR for *dfrA1*, *dfrA5*, *dfrA7*, *dfrA8*, *dfrA12*, *dfrA13* and *dfrA17* was first performed (Lee *et al.*, 2001b). Southern hybridization of the PFGE gel with a *dfrA1* probe showed that all 122 *S. sonnei* isolates carried *dfrA1* in their chromosomes (Table 3, Fig. 1B). To characterize the association of *dfrA1* genes with Tn7, *intI2* and

Table 3. Characterization of non-transferable antimicrobial resistance in *S. sonnei* isolates

For abbreviations, see Table 1.

Year isolated	No. of isolates	Antimicrobial resistance	Antimicrobial resistance genes located in:	
			Chromosome	Non-transferable R plasmid
1991	5	TcSmSuApTpKm	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	1	SmSuApTpKm	Tn7 (<i>dfrA1-sat-aadA1</i>)	—
1992–1995	5	TcSmSuTp	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
1997	1	TcSmSuTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
1998	28	TcSmSuTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	1	TcSmSuApTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	1	SmTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	—
1999	55	TcSmSuTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	6	SmTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	—
	5	TcSmSuApTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	4	TcSmSuApTpKmNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	3	CmTcSmSuTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	1	TcSmSuApCpTpGmNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	1	TcSmSuTpKmGmNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
2000	3	TcSmSuTpNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	1	TcSmSuApTpKm	Tn7 (<i>dfrA1-sat-aadA1</i>)	<i>tetA</i> , <i>strA-strB</i> , <i>sul1</i>
	1	TcSmSuApTpKmNa	Tn7 (<i>dfrA1-sat-aadA1</i>)	—

gene cassette regions of class 2 integrons, a 440 bp fragment of *intI2* and 2224 bp of a class 2 integron, were amplified and sequenced. All *S. sonnei* isolates carried *dfrA1* as a gene cassette of Tn7, *dfrA1-sat-aadA1*.

Of the 122 *S. sonnei* isolates tested, 113 (92.6%) isolates carried 8.4 kb of non-transferable R-plasmid DNA, as demonstrated by a plasmid profile and conjugation experiment (Fig. 3A, Table 3). To characterize the 8.4 kb non-transferable R plasmid, plasmid DNA was extracted from *S. sonnei* H111, which was resistant to tetracycline, streptomycin, sulfamethoxazole, trimethoprim and nalidixic acid, and introduced into *E. coli* DH5 α by transformation. The transformant carrying the 8.4 kb non-transferable R plasmid exhibited resistance to tetracycline, streptomycin and sulfamethoxazole, which were mediated by *tetA*, *strA-strB* and *sul1*, respectively. The antimicrobial resistance genes and restriction map of the 8.4 kb of non-transferable R plasmid in the current study were identical to the GenBank entry for pKKTET7 of the *S. sonnei* isolate from 1998 in Korea (AF497970). To determine whether 113 *S. sonnei* isolates carried *tetA*, *strA-strB* and *sul1* in the 8.4 kb of non-transferable R plasmid, PCR amplification and Southern hybridization were performed, which revealed that *tetA*, *strA-strB* and *sul1* were present and encoded in the 8.4 kb of non-transferable R plasmid (Fig. 3B, Table 3). It was concluded that the 8.4 kb of non-transferable R plasmid was responsible for the high prevalence of resistance to these agents in *S. sonnei* isolates in Korea.

Resistance to nalidixic acid first appeared in a *S. sonnei* isolate in 1997, and then in all *S. sonnei* isolates from 1998 and 1999 (Table 1). However, nalidixic acid-susceptible *S. sonnei* isolates reappeared in a defined outbreak of shigellosis in 2000.

Fig. 3. Plasmid profiles of *S. sonnei* isolates (A) and Southern blot of gel hybridized with *tetA* gene (B). Lanes: M, size marker from *E. coli* KE327; 1, H008 from 1992; 2, H002 from 1991; 3, H012 from 1995; 4, H022 from 1998; 5, H025 from 1998; 6, H101 from 1999; 7, H111 from 1999; 8, H003 from 1991; 9, H152 from 2000; 10, H043 from 1998; 11, H115 from 1999; 12, H151 from 2000; 13, H210 from 1999.

DISCUSSION

The current study demonstrated that *S. sonnei* isolates have acquired resistance to ampicillin through conjugative R plasmids carrying *bla*_{TEM-1}, while the 8.4 kb of non-transferable R plasmid carrying *tetA*, *strA-strB* and *sul1* is responsible for resistance to tetracycline, streptomycin and sulfamethoxazole. Resistance genes for ampicillin, gentamicin and kanamycin, except for one ampicillin-resistance gene, were all located in the conjugative R plasmids. However, resistance genes for trimethoprim and sulfamethoxazole were mainly located in Tn7 in the chromosome and in the 8.4 kb non-transferable R plasmid, respectively. Some of the resistance genes for trimethoprim and sulfamethoxazole were also found in the conjugative R plasmids as a gene cassette of class 1 integrons.

Bacteria can acquire antimicrobial resistance when they are confronted with antibiotic selective pressures. The most commonly encountered resistance mechanism among *Enterobacteriaceae* is the acquisition of antimicrobial resistance genes through mobile genetic elements, including R plasmids and transposons. Ampicillin has been the most commonly used antibiotic for treatment of shigellosis in Korea during the last two decades. Resistance to ampicillin was found in all six *S. sonnei* isolates from 1991, but not in the isolates from 1992 to 1997; thereafter, resistance to ampicillin reappeared during the outbreak period 1998–2000 (Table 1). All ampicillin-resistant *S. sonnei* isolates carried the conjugative R plasmids harbouring *bla*_{TEM-1}. This finding indicates that *S. sonnei* can acquire resistance to commonly prescribed antibiotics through conjugative R plasmids.

Since the first emergence of resistance to trimethoprim in *S. sonnei* isolates from 1978 in Korea (Chun & Seol, 1979), resistance to trimethoprim has been detected in 94% of *S. sonnei* isolates from the 1980s and in 100% of isolates from the 1990s and 2000s. Accordingly, trimethoprim combined with sulfamethoxazole (co-trimoxazole) is no longer recommended in Korea for treatment of shigellosis. In the current study, resistance to trimethoprim was mediated by *dfrA1* and/or *dfrA12*. *dfrA1* was located in the chromosome as a gene cassette of Tn7, while *dfrA12* was found as a gene cassette of class 1 integrons in the conjugative R plasmids. Tn7 was not detected in the *S. sonnei* isolates from the 1980s, while Tn7 was detected in all *S. sonnei* isolates from the 1990s and 2000s (Kim *et al.*, 2002). This suggests that the *S. sonnei* strains carrying Tn7 in the chromosome were introduced at this time and widely disseminated in all parts of Korea. To date, five class 1 integrons carrying *dfr* genes, *dfrA1*-unknown ORF, *dfrA1-aadA2*, *dfrA12-orfF-aadA2*, *dfrA17-aadA5* and *aacA4-catB4-dfrA1*-unknown gene, have been detected among *Enterobacteriaceae* in Korea (Yu *et al.*, 2003). However, gene cassettes of *dfrA12-orfF-aadA2* were identified in the *S. sonnei* isolates tested. It would seem that *S. sonnei* has selected class 1 integrons carrying *dfrA12-orfF-aadA2*, thereby accounting for the occurrence of *dfrA12*.

In addition to trimethoprim, sulfamethoxazole is one of the traditional antibiotics used to treat bacterial infection. Since resistance to sulfamethoxazole was reported to be found in both *S. sonnei* and *Shigella flexneri* isolates from the early 1970s in Korea (Oh & Chun, 1977), single therapy with sulfamethoxazole has not been recommended to treat shigellosis. In the current study, 94% of *S. sonnei* isolates were resistant to sulfamethoxazole, which was mediated by *sul1* and/or *sul2*. Of the 115 sulfamethoxazole-resistant *S. sonnei* isolates, *sul1* was detected in the 114 *S. sonnei* isolates, while *sul2* was detected in six isolates carrying conjugative R plasmids. Accordingly, resistance to sulfamethoxazole was mainly mediated by *sul1*, which was located in the 8.4 kb of non-conjugative R plasmid.

We inferred that Korean endemic *S. sonnei* strains disappeared in the late 1980s and that a new *S. sonnei* clone was first introduced to Korea between the late 1980s and early 1990s (Jeong *et al.*, 2003; Kim *et al.*, 2002; Oh *et al.*, 2003). The new clone of *S. sonnei* exhibited biotype g and a characteristic PFGE pattern (Fig. 1A). They carried 8.4 kb of non-transferable R plasmid and were commonly resistant to tetracycline, streptomycin, sulfamethoxazole, trimethoprim and nalidixic acid. Among the six *S. sonnei* isolates from 1991, five carried 8.4 kb of non-transferable plasmid DNA. This was the first appearance of the 8.4 kb of non-transferable plasmid in the new clone of *S. sonnei*. Thereafter, all *S. sonnei* isolates tested, except for eight isolates, carried this non-transferable R plasmid. This suggests that the 8.4 kb of non-transferable plasmid is genetically stable in *S. sonnei* strains.

An increase in the use of first-line antibiotics, such as ampicillin and co-trimoxazole, for shigellosis might be responsible for the acquisition of resistance to these antibiotics in *S. sonnei* isolates. The acquisition of conjugative R plasmids carrying *bla*_{TEM-1} was responsible for resistance to ampicillin. Tn7 in the chromosome was responsible for resistance to trimethoprim and streptomycin. The acquisition of the 8.4 kb of non-transferable R plasmid was responsible for resistance to tetracycline, streptomycin and sulfamethoxazole. Accordingly, combinations of three genetic repertoires, conjugative R plasmids, Tn7 and the 8.4 kb of non-transferable R plasmid, can cover resistance to the antibiotics generally used for treatment of shigellosis. Because *S. sonnei* has undergone evolutionary changes in antimicrobial resistance over time, an appropriate antimicrobial therapy will be necessary for treatment of shigellosis to prevent the emergence of a new type of antimicrobial resistance or multiresistance. The diversity of ESBL genes in *S. sonnei* strains is an example of the evolutionary changes in antimicrobial resistance.

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REFERENCES

- Birnboim, I. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7, 1513–1523.
- Chun, D. & Seol, S. Y. (1979). Drug resistance of *Shigella* and *Salmonella* and the inhibition and elimination of drug resistance. *J Korean Soc Microbiol* 14, 27–37.
- Ewing, W. H. (1986). *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th edn. New York: Elsevier.
- Im, Y. H. & Choi, K. D. (1961). Studies on the *Shigella* isolated in Southern Korea with reference to non-*Shigella* strains possessing *Shigella* antigens. *Showa Med J* 20, 1–8.
- Jeong, Y. S., Lee, J. C., Kang, H. Y. & 7 other authors (2003). Epidemiology of nalidixic acid resistance and TEM-1- and TEM-52-mediated ampicillin resistance of *Shigella sonnei* isolates obtained in Korea between 1980 and 2000. *Antimicrob Agents Chemother* 47, 3719–3723.
- Kim, K. S., Oh, J. Y., Jeong, Y. W., Cho, J. W., Park, J. C., Cho, D. T. & Lee, J. C. (2002). Epidemiological typing and characterization of *df*r genes of *Shigella sonnei* isolates in Korea during the last two decades. *J Microbiol Biotechnol* 12, 106–113.
- Kim, S., Kim, J., Kang, Y., Park, Y. & Lee, B. (2004). Occurrence of extended-spectrum β -lactamases in members of the genus *Shigella* in the Republic of Korea. *J Clin Microbiol* 42, 5264–5269.
- Lee, J. C., Oh, J. Y., Cho, J. W., Park, J. C., Kim, J. M., Seol, S. Y. & Cho, D. T. (2001a). The prevalence of trimethoprim-resistance-conferring dihydrofolate reductase genes in urinary isolates of *Escherichia coli* in Korea. *J Antimicrob Chemother* 47, 599–604.
- Lee, J. C., Oh, J. Y., Kim, K. S., Jeong, Y. W., Cho, J. W., Park, J. C., Seol, S. Y. & Cho, D. T. (2001b). Antimicrobial resistance of *Shigella sonnei* in Korea during the last two decades. *APMIS* 109, 228–234.
- National Committee for Clinical Laboratory Standards (1997). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*. Approved standard M7-A4. Wayne, PA: National Committee for Clinical Laboratory Standards.
- National Institute of Health in Korea (2005). *The Annual Frequency of Shigellosis in Korea*. http://dis.cdc.go.kr/statistics/Statistics_Disease_Year.asp (in Korean).
- Oh, S. Y. & Chun, D. (1977). Antimicrobial resistance and R plasmids in *Shigella*. *Korean J Internal Med* 20, 591–596.
- Oh, J. Y., Kim, K. S., Jeong, Y. W., Cho, J. W., Park, J. C. & Lee, J. C. (2002). Epidemiological typing and prevalence of integrons in multiresistant *Acinetobacter* strains. *APMIS* 110, 247–252.
- Oh, J. Y., Yu, H. S., Kim, S. K., Seol, S. Y., Cho, D. T. & Lee, J. C. (2003). Changes in patterns of antimicrobial susceptibility and integron carriage among *Shigella sonnei* isolates from southwestern Korea during epidemic periods. *J Clin Microbiol* 41, 421–423.
- Pai, H., Choi, E., Lee, H., Hong, J. Y. & Jacoby, G. A. (2001). Identification of CTX-M-14 extended-spectrum β -lactamase in clinical isolates of *Shigella sonnei*, *Escherichia coli*, and *Klebsiella pneumoniae* in Korea. *J Clin Microbiol* 39, 3747–3749.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 33, 2233–2239.

Voogd, C. E., Schot, C. S., van Leeuwen, W. J. & van Klingeren, B. (1992). Monitoring of antibiotic resistance in *Shigella* isolated in the Netherlands 1984–1989. *Eur J Clin Microbiol Infect Dis* **11**, 164–167.

White, P. A., Mclver, C. J., Deng, Y. M. & Rawlinson, W. D. (2000). Characterization of the two gene cassettes, *aadA5* and *dfrA17*. *FEMS Microbiol Lett* **182**, 265–269.

White, P. A., Mclver, C. J. & Rawlinson, W. D. (2001). Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob Agents Chemother* **45**, 2658–2661.

Yu, H. S., Lee, J. C., Kang, H. Y. & 9 other authors (2003). Changes in gene cassettes of class 1 integrons among *Escherichia coli* isolates from urine specimens collected in Korea during the last two decades. *J Clin Microbiol* **41**, 5429–5433.