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

Sung Yong Seol, Yong Tae Kim, Young Sook Jeong, Jae Young Oh, Hee Young Kang, Dong Chan Moon, Jungmin Kim, Yoo Chul Lee, Dong Taek Cho and Je Chul Lee

Department of Microbiology, Kyungpook National University School of Medicine, 101, Dongin-dong, Jung-gu, Daegu, 700-422, Korea

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 conjugal R plasmids carrying blTEM-1. In conclusion, *S. sonnei* acquired antimicrobial resistance to commonly prescribed antibiotics through the horizontal transfer of conjugal R plasmids, while the genetic stability of transposon and non-transferable R plasmids was responsible for resistance to traditional antibiotics.

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

<table>
<thead>
<tr>
<th>Year isolated</th>
<th>Isolation area</th>
<th>No. of isolates</th>
<th>Antimicrobial resistance</th>
<th>No. of trans-conjugants</th>
<th>Transferrable antimicrobial resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1991</td>
<td>Unknown (6)</td>
<td>5</td>
<td>TcSmSuApTpKm</td>
<td>5</td>
<td>SmSuApTpKm (1), ApKm (4)</td>
</tr>
<tr>
<td>1992–1995</td>
<td>Unknown (3)/Seoul (1)/Kyungpook (1)</td>
<td>5</td>
<td>TcSmSuTp</td>
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<td>–</td>
</tr>
<tr>
<td>1997</td>
<td>Kyungpook (1)</td>
<td>1</td>
<td>TcSmSuTpNa</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>1998</td>
<td>Kyungpook (28)</td>
<td>28</td>
<td>TcSmSuTpNa</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>1999</td>
<td>Kyungpook (28)/Chunnam (27)</td>
<td>55</td>
<td>TcSmSuTpNa</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Kyungpook (6)</td>
<td>6</td>
<td>TcSmSuTpNa</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Kyungpook (3)/Chunnam (2)</td>
<td>5</td>
<td>TcSmSuTpNa</td>
<td>5</td>
<td>SmSuApTp (3), TcSmSuAp (1), TcSmSu (1)</td>
</tr>
<tr>
<td></td>
<td>Chunnam (4)</td>
<td>4</td>
<td>TcSmSuApTpKmNa</td>
<td>4</td>
<td>SmSuApKm (1), ApKm (3)</td>
</tr>
<tr>
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<td>Chunnam (3)</td>
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</tr>
<tr>
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<td>SmSuApCpTpGm (1)</td>
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<td>SmSuTpKmGm (1)</td>
</tr>
<tr>
<td>2000</td>
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<td>TcSmSuTpNa</td>
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<td>–</td>
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<td>1</td>
<td>TcSmSuApTpKmNa</td>
<td>1</td>
<td>TcSmSuApKm (1)</td>
</tr>
</tbody>
</table>

Abbreviations: Tc, tetracycline; Sm, streptomycin; Su, sulfamethoxazole; Ap, ampicillin; Tp, trimethoprim; Km, kanamycin; Na, nalidixic acid; Cm, chloramphenicol; Gm, gentamicin; Cp, cepheprazone.

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), trimethoprim (≥16 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 R684 RifR and J53 AzR 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 Smal 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 hep8/hep9 and hep7A/hep7B, respectively (White et al., 2000, 2001). To determine identical arrays of gene cassettes, same-sized amplicons were digested with Rsal 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 dyeoxy 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-CAGTGGAAAGC-3’) (Jeong et al., 2003).

PCR amplification of antimicrobial resistance genes. PCR was performed to amplify a specific antimicrobial resistance gene for...
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
Integrons were found in any of the transconjugants. Among the five conjugative R plasmids carrying class 1 integrons, four carried a gene cassette of \(\text{dfrA12-orfF-aadA2}\) and one carried \(\text{aadA1}\). A class 1 integron carrying \(\text{dfrA12-orfF-aadA2}\) was found in \(S.\) \(\text{sonnei}\) isolates from 1991, 1999 and 2000, while a class 1 integron carrying \(\text{aadA1}\) 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 \(\text{tetA}\), \(\text{aph}\), \(\text{bla}\), \(\text{strA-strB}\) and \(\text{dfrA1}\), respectively (Table 2). Resistance to ceftazidime was mediated by \(\text{bla}\) in one \(S.\) \(\text{sonnei}\) isolate from 1999 (Jeong et al., 2003). Resistance to sulfamethoxazole was mediated by \(\text{sul1}\) in the five class 1 integron-carrying R plasmids, and \(\text{sul2}\) in the four R plasmids that did not contain class 1 integrons. Resistance to streptomycin was mediated by \(\text{strA-strB}\), \(\text{aadA2}\) or \(\text{aadA1}\).

### Molecular characterization of non-transferable antimicrobial resistance

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

![Fig. 2. Restriction endonuclease analysis of R plasmids extracted from transconjugants by EcoRI (A) and Smal (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 H285.](image-url)
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 DH5α 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.

DISCUSSION

The current study demonstrated that S. sonnei isolates have acquired resistance to ampicillin through conjugative R plasmids carrying blaTEM-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 blaTEM-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. Therefore, 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 blaTEM-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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