Characterization of *Shigella sonnei* isolates from travel-associated cases in Japan

Hidemasa Izumiya,1 Yuki Tada,2 Kenichiro Ito,2 Tomoko Morita-Ishihara,1 Makoto Ohnishi,1 Jun Terajima1 and Haruo Watanabe1

Correspondence
Hidemasa Izumiya
izumiya@nih.go.jp

1Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan
2Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan

*Shigella sonnei* infection in industrialized countries is often associated with foreign travel. A total of 195 *S. sonnei* isolates in Japan, isolated from cases associated with foreign travel, were analysed by biotyping and molecular typing using PFGE and multilocus variable-number tandem-repeat analysis (MLVA); their antimicrobial susceptibilities were also evaluated. The isolates were from 26 countries, most of which were Asian. Molecular typing revealed a correlation among the genotypes, biotypes and their geographical areas of origin. The isolates were classified into two biotypes, a and g. Biotype g isolates (*n* = 178) were further divided into distinct clusters mainly on the basis of their geographical areas of origin by both PFGE and MLVA. Isolates from South Asian countries constituted one of the distinct clusters. Biotype g isolates from countries other than South Asia constituted other distinct clusters. Most of the isolates from other countries and continents, excluding the South Asian countries, were included in one major cluster by PFGE analysis. However, by MLVA, they were further divided into minor subclusters mainly on the basis of their countries of origin. MLVA was also demonstrated to be useful in molecular epidemiological analysis, even when only seven loci were applied, resulting in a high resolution with Simpson’s index of diversity (*D*) of 0.993. A core drug-resistance pattern of streptomycin, sulfisoxazole, tetracycline and trimethoprim–sulfamethoxazole was observed in 108 isolates, irrespective of their geographical areas of origin, but the frequency of resistance to nalidixic acid was high among the South Asian and East Asian isolates. Two isolates from China and India were resistant to cefotaxime and harboured the *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub> genes, respectively; these isolates were also resistant to nalidixic acid, which is a matter of concern in terms of shigellosis treatment. Use of a combination of methods was found to be effective for epidemiological investigation in the case of *S. sonnei* infection.

**INTRODUCTION**

*Shigella* infection is one of the major public health problems worldwide. An estimated 164.7 million *Shigella* episodes have been identified in two-thirds of young children (World Health Organization, 2006). Shigellosis occurs mainly in developing countries due to poor hygiene and limited access to clean drinking water (Samosis *et al.*, 1994; Simchen *et al.*, 1991), whereas in industrialized countries it is mainly because of travel to developing countries and exposure to contaminated foods and/or food handlers.

Shigellosis is a notifiable disease in Japan and the annual incidence of *Shigella* infection is approximately 500. There are four *Shigella* species, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*. *S. sonnei* is the dominant among these, accounting for about four-fifths of the shigellosis cases in Japan, and most of these cases are associated with foreign travel (National Institute of Infectious Diseases, 2006). Outbreaks are sometimes associated with imported foods, such as oysters (Terajima *et al.*, 2004).

Molecular typing methods are powerful tools for epidemiological investigation of *Shigella* infections as well as other bacterial infections such as those caused by Shiga-toxin-producing *Escherichia coli* O157 : H7. PFGE is the gold standard method among the molecular typing methods with a standardized PulseNet protocol, which makes it possible to compare bacterial DNA fingerprints among laboratories, even internationally (Gerner-Smidt *et al.*, 2006; Ribot *et al.*, 2006). Nevertheless, more powerful and easier methods have been devised. Multilocus variable-number tandem-repeat analysis (MLVA) is a newly devised...
molecular typing method has been applied to several bacterial pathogens, including S. sonnei (Liang et al., 2007; van Belkum, 2007). In this study, we examined S. sonnei isolates from foreign-travel-associated cases in Japan by molecular typing, biotyping and antimicrobial susceptibility testing to explore the recent epidemiological trends of this bacterial agent.

METHODS

Bacterial strains. A total of 195 S. sonnei isolates were collected from local public health institutes and Narita Air Port Quarantine between 1998 and 2005 and analysed in this study. Although shigellosis is a notifiable disease, not all of the isolates were sent to our laboratory to be analysed. In this study, isolates from travel-associated cases, where a history of foreign travel before the onset of illness was obtained by means of a questionnaire, were selected from our collection of isolates from 26 countries. The South Asian countries included India (n=64), Nepal (n=1) and Sri Lanka (n=1); the South-east Asian countries comprised Indonesia (n=34), Vietnam (n=14), Thailand (n=9), the Philippines (n=7) and Cambodia (n=5); and the East Asian countries were China (n=12), Korea (n=2), North Korea (n=2) and Taiwan (n=1). For the other areas, 11 isolates were from the USA, eight were from Peru, six were from Mexico, five were from Egypt, two each were from Morocco, Senegal and Turkey, and one each was from Ethiopia, Guatemala, New Caledonia, Russia, Spain, Syria and Uzbekistan. Nine isolates were associated with an outbreak due to travel to Hawaii in 2004 (Terajima et al., 2007).

The ages of patients ranged from 4 to 63 (median 28), and the majority of the patients belonged to age groups 20–29 (57 % and 28 %, respectively). Males accounted for 44 %.

Biochemical typing. A standard method of detecting rhamnose fermentation was used for biochemical typing. Biotypes were designated according to a scheme described previously (Nastasi et al., 1993).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using a disc diffusion method as described previously according to the standards outlined by the Clinical and Laboratory Standards Institute (CLSI, 2006). E. coli ATCC 25922 was used as the quality-control strain. The discs used were ampicillin, chloramphenicol, streptomycin, tetracycline, ciprofloxacin, kanamycin, cefotaxime, trimethoprim–sulfamethoxazole, gentamicin, nalidixic acid and sulfisoxazole (Becton Dickinson Microbiology Systems). Class 1 integrons were screened by using intI1 (5’-ACATGTGATGGAGACGACGA-3’) and intI2 (5’-ATTTCGTCCTGAGCCTCT-3’) as primers (Ploy et al., 2000). Two cefotaxime-resistant isolates were screened further for genes encoding cefotaxime resistance and found to be cefotaxime-resistant.

Table 1. MLVA primers, number of alleles and index of diversity (D)

<table>
<thead>
<tr>
<th>Locus*</th>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Fluorescent dye</th>
<th>No. of alleles</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1</td>
<td>SS1-F</td>
<td>TTGGCAGTACACCTCACCTCG*</td>
<td>6-FAM</td>
<td>14</td>
<td>0.914</td>
</tr>
<tr>
<td>SS1</td>
<td>SS1-R</td>
<td>GGTCGGGCGGTTAATATCCT*</td>
<td>NED</td>
<td>25</td>
<td>0.839</td>
</tr>
<tr>
<td>SS3</td>
<td>SS3-F</td>
<td>CTGGGAGGTGAAAGACGGAGGA*</td>
<td>PET</td>
<td>27</td>
<td>0.941</td>
</tr>
<tr>
<td>SS3</td>
<td>SS3-R</td>
<td>ATGCCGGGCAACGGTTCTTTT*</td>
<td>VIC</td>
<td>12</td>
<td>0.850</td>
</tr>
<tr>
<td>SS6</td>
<td>SS6-F</td>
<td>GAGTCGGTAAACCGCTTGTT*</td>
<td>VINA600A</td>
<td>9</td>
<td>0.712</td>
</tr>
<tr>
<td>SS6</td>
<td>SS6-R</td>
<td>GGGAATTAGAGGGCGATTTTTT*</td>
<td>VIC</td>
<td>12</td>
<td>0.850</td>
</tr>
<tr>
<td>SS9</td>
<td>SS9v-F</td>
<td>GTGGTGTAACCGCTGGCACA</td>
<td>VIC</td>
<td>12</td>
<td>0.850</td>
</tr>
<tr>
<td>SS9</td>
<td>SS9v-R</td>
<td>CGCGGAGTCGCCGGAGATAC</td>
<td>VINA600A</td>
<td>9</td>
<td>0.712</td>
</tr>
<tr>
<td>SS10</td>
<td>SS10v-F</td>
<td>GGGGGAGATCGATTATTTGA</td>
<td>VINA600A</td>
<td>9</td>
<td>0.712</td>
</tr>
<tr>
<td>SS10</td>
<td>SS10-R</td>
<td>TGATAGACGGTTATGGCGATAATGTGAAC</td>
<td>PET</td>
<td>8</td>
<td>0.732</td>
</tr>
<tr>
<td>SS11</td>
<td>SS11v-F</td>
<td>GCATTCTGTTTTTTTATCGAC</td>
<td>6-FAM</td>
<td>5</td>
<td>0.681</td>
</tr>
<tr>
<td>SS11</td>
<td>SS11v-R</td>
<td>CTGGCAGAGCGACGAGGAC</td>
<td>6-FAM</td>
<td>5</td>
<td>0.681</td>
</tr>
<tr>
<td>SS13</td>
<td>SS13v-F</td>
<td>CCATTGTGACATCCGGTTGT</td>
<td>6-FAM</td>
<td>5</td>
<td>0.681</td>
</tr>
<tr>
<td>SS13</td>
<td>SS13-R</td>
<td>GTCCTCACAAGGATGCTGTGC</td>
<td>6-FAM</td>
<td>5</td>
<td>0.681</td>
</tr>
</tbody>
</table>

*According to Liang et al. (2007).

PFGE. PFGE was performed according to the PulseNet protocol using the Salmonella Braenderup H9812 strain as a standard for normalization (Ribot et al., 2006). DNA was digested with XbaI and separated using a CHEF DR III apparatus (Bio-Rad Laboratories) under the following conditions: switching time from 2.2 to 54.2 s at 6 V cm\(^{-1}\) for 20 h at 14 °C. The resulting profiles were analysed as described previously (Izumiya et al., 2005a).

MLVA. MLVA was performed according to a previous study with modifications (Liang et al., 2007). The following eight loci were selected: SS1, SS3, SS6, SS9, SS10, SS11, SS13 and SS23. Some of the primer sequences were modified to reduce the number of reaction mixtures. Primers used in this study are shown in Table 1. PCR was performed in a single mixture, except in the case of SS23. The PCR mixture contained 0.2 μM each primer, DNA template and 1 × multiplex PCR mixture (Qiagen KK). PCR conditions were as follows: initial denaturation at 95 °C for 15 min; 35 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 90 s and extension at 72 °C for 60 s; and final extension at 72 °C for 10 min.

The resulting PCR products were diluted and separated with an ABI 3130 genetic analyser using Gene Scan LIZ 600 (Applied Biosystems) as the size standard. Their sizes were converted to the repeat copy number using GeneMapper software. The data were incorporated into BioNumerics software and analysed as described previously (Hyytia-Trees et al., 2006). Simpson’s index of diversity (D) and 95 % confidence intervals (CI) were calculated according to formulae described previously (Grundmann et al., 2001).

Downloaded from www.microbiologyresearch.org by IP: 54.70.40.11
On: Tue, 20 Nov 2018 01:01:13
extended-spectrum β-lactamases as described previously (Izumiya et al., 2005b).

RESULTS AND DISCUSSION

Biotype
Recently, there was a report on infection caused by S. sonnei biotype g (negative for rhamnose fermentation) (Mammina et al., 2006). We examined the biotype distribution of the isolates in this study, and found 178 and 17 of 195 isolates to be biotype g and a (positive for rhamnose fermentation) isolates, respectively. Almost all of the biotype a strains were isolated from cases with a history of travel to South-east Asia, namely the Philippines (n=7), Indonesia (n=5) and Vietnam (n=2). All of the isolates from the Philippines in this study were biotype a isolates.

Molecular typing
Subtyping of S. sonnei isolates using either PFGE or MLVA resulted in two gross clusters; this result was consistent with the biotype grouping of the isolates. The biotype a and g isolates were clearly assigned to different clusters by PFGE and MLVA, irrespective of their geographical areas of origin (Figs 1 and 2). For example, isolates from Indonesia, of which five and 29 were biotypes a and g, respectively, were assigned to different clusters on the basis of their biotypes. This suggests that the fermentation of rhamnose is a relevant epidemiological marker representative of certain genotypes.

Whereas subtyping on the basis of rhamnose fermentation yielded only two groups (biotypes a and g), application of PFGE and MLVA aided in further differentiation of the biotype g isolates. One major cluster (Fig. 1, cluster gS) was identified by PFGE; this cluster included all of the isolates from South Asia (n=66). Clusters of isolates from South Asia were also identified and further divided into two clusters (Fig. 2, clusters gS1 and gS2) by MLVA. Another cluster (cluster gP) flanking cluster gS (Fig. 1) and cluster gS2 (Fig. 2) was identified by PFGE and MLVA, respectively. Cluster gP consisted only of isolates from Peru. This suggests that S. sonnei strains in these areas are unique and circulate within limited geographical areas.

Not all clusters contained isolates from a single geographical region. One major cluster identified by PFGE consisted of 90 isolates from 18 countries other than South Asia when the cut-off value was set at 90% similarity (Fig. 1, cluster gM). Indistinguishable isolates from up to five countries were also observed by PFGE. Most of these isolates are likely to be unrelated geographically and temporally, a clear example being the isolates associated with an outbreak due to travel to Hawaii in 2004 (Terajima et al., 2006). Five of the nine outbreak-related isolates were assigned to or located near a subcluster (Fig. 1, subcluster h). This subcluster also contained epidemiologically unrelated isolates from China, Korea, Thailand and Vietnam in the years 2001–2003. The remaining four outbreak-related isolates were located far from the subcluster (Fig. 1). Thus, the epidemiologically related isolates were segregated into different subclusters by PFGE analysis. In contrast, the nine Hawaii outbreak-related isolates were grouped together by MLVA, although not by
PFGE (Fig. 2). Thus, the results of MLVA were more consistent with the epidemiological information than the PFGE results.

A low similarity overall was observed by MLVA, and the diversity was greater than that observed by PFGE; furthermore, most of the isolates were classified according to country by MLVA. For example, 24 of 29 biotype g isolates from Indonesia were located within a relatively large subcluster by MLVA; however, they were dispersed in clusters by PFGE analysis (Fig. 2). Another example is that of the MLVA complexes. A total of 18 complexes were identified by MLVA where the isolates differed by a single locus (single-locus variants) (Fig. 2 and Table 2). Thirteen of the 18 complexes included isolates from single countries, whilst three of the remaining complexes included isolates from two neighbouring countries (complexes 2, 4 and 8). Four of the five isolates in complex 6 were from the same country, namely Vietnam. These results suggest that cluster analysis by PFGE is suitable for rough classification, but MLVA is more effective for precise characterization of S. sonnei, especially in inter-outbreak investigation as well as intra-outbreak investigation.

Table 1 shows the number of alleles and $D$ value for each MLVA locus identified in this study. The allelic numbers and $D$ values ranged from 5 to 27 and from 0.147 to 0.941, respectively. Overall, 195 isolates were divided into 175 types on the basis of MLVA with an overall $D$ value of 0.993 (CI 0.992–0.995). By using PFGE, 138 types were identified with a $D$ value of 0.983 (CI 0.946–1.02). This indicates that MLVA has a higher resolution power than PFGE analysis. Among the eight loci, SS23 showed the lowest $D$ value in this study. The number of types and overall $D$ values were unchanged when the remaining seven
Antimicrobial resistance

Frequencies of the resistance to test antimicrobial agents were as follows: streptomycin (S), 153/195 (78%); sulfisoxazole (Su), 122/195 (63%); trimethoprim-sulfamethoxazole (Sx), 122/195 (63%); tetracycline (T), 118/195 (61%); nalidixic acid (N), 75/195 (38%); ampicillin (A), 20/195 (10%); chloramphenicol (C), 10/195 (5%); cefotaxime, 2/195 (1%); gentamicin, 2/195 (1%); kanamycin, 1/195 (1%); and ciprofloxacin, 0/195 (0%). The resistance types SSuTSxN (n=51) and SSuTSx (n=48) were dominant. The resistance types containing a core type SSuTSx were detected in a total of 108 isolates. None were resistant to ciprofloxacin, but the frequency of resistance to N was moderate. Isolates from India constituted the major proportion of the N-resistant isolates (59/64; 92%) followed by isolates from China (10/12, 83%), Vietnam (2/14, 14%), Korea (2/2, 100%), Thailand (1/9, 11%) and Nepal (1/1, 100%). These results indicate that SSuTSx is the major core resistance type and that the frequency of N resistance is high in South Asia and East Asia and low in South-east Asia. Although no ciprofloxacin-resistant isolates were identified in this study, N resistance is a relevant marker of decreased susceptibility to ciprofloxacin (Cheasty et al., 2004; Hirose et al., 2005) and could result in treatment failure in other enteric bacterial infections (Aarestrup et al., 2003). Much attention should be paid to treating patients who develop shigellosis after travelling to these areas.

Fifteen isolates were positive for class 1 integrons (eight from Peru, three from China, two from Indonesia, and one each from Mexico and Sri Lanka). All of the isolates from Peru were positive for class 1 integrons, and seven had a common resistance type, namely ACSSuTSx. Two cefotaxime-resistant isolates were also identified in this study from China and India, and the bla CTX-M-14 and blaCTX-M-15 genes were identified in these isolates, respectively. Both were also resistant to N and shared the core resistance type SSuTSx. This raises another public health issue with regard to shigellosis treatment.

In this study, we revealed the current trends in epidemiology of isolates from travel-associated cases of S. sonnei in Japan, and the correlations among molecular types, biotypes, resistance types and their geographical areas of origin. Our study also suggests that MLVA using the seven loci applied in this study is useful for precise investigation, for example in outbreaks, whilst PFGE is suitable for rough classification of isolates. Infection with S. sonnei is associated not only with foreign travel but also with consumption of imported foods in industrialized countries (Lewis et al., 2009). Thus, use of a combination of methods is effective for epidemiological investigation.

ACKNOWLEDGEMENTS

We thank all of the municipal and prefectoral local public health institutes and Narita Airport Quarantine for providing us with the Shigella isolates. We thank Ms Kadumi Mori, Ms Nobuko Takai and Ms Tamayo Kudo for their technical assistance. This work was supported partly by grants-in-aid from the Ministry of Health, Labour and Welfare of Japan (H18-Shokuhin-Ippan-003, H20-Shinko-Ippan-013, H20-Shinko-Ippan-015, and International Health Cooperation Research 18C-5).

REFERENCES


