Epidemiological Evidence of Multidrug-Resistant *Shigella sonnei*
Colonization in India by Sentinel Surveillance in a Japanese Quarantine Station

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Abstract

We applied a previously reported method to clarify whether a multidrug-resistant *Shigella* colonizes in a south Asian country. At Kansai Airport Quarantine Station, stool samples were collected from overseas travelers who reported a history of diarrhea. *Shigella* strains were isolated, ranging from 53 to 106 (average, 82) isolates/year (2001-2005), and almost 80% of the isolates were *Shigella sonnei*. The most frequent country of origin was India. Strains from the country of the most frequent origin were studied by antimicrobial susceptibility testing. Resistance to tetracycline, sulfamethoxazole-trimethoprim and nalidixic acid was observed at the highest frequency: in 23 of the 25 strains isolated in 2001, 5 of the 13 strains isolated in 2002, and 16 of the 19 strains isolated in 2005. Strains showing the most prevalent multidrug-resistance pattern were analyzed by pulsed-field gel electrophoresis (PFGE). The PFGE profiles showed that 27 of the 44 strains isolated in 2001, 2002, and 2005 were identical in PFGE pattern, as determined using two restriction enzymes. We concluded that a multidrug-resistant *Shigella sonnei* colonizes in a south Asian country.


Introduction

Shigellosis is a bacillary dysentery caused by *Shigella* spp., and is endemic throughout the world. Epidemics of shigellosis have been reported from many Asian countries, such as India and Bangladesh. Bacillary shigellosis is one of the diseases that is imported into Japan by overseas travelers. Japanese quarantine stations (JQSs) check for overseas travelers with diarrhea at airports and ports. Taniguchi et al. reported that the international prevalence pattern of *Shigella sonnei* is inferred by monitoring isolates from passengers arriving at international airports in Japan.
The JQS at Kansai International Airport reports 50 to 100 Shigella isolates annually, 80% of which are S. sonnei. In the epidemiological analysis of Shigella spp., the number of drug-resistant isolates from overseas travelers has increased as compared with that before 1979 in the JQS of an airport in Osaka area.

Concerning drug-resistance, mutations of chromosomal DNA, such as of gyrA, determining nalidixic acid resistance, and plasmids or transposons responsible for tetracycline, sulfamethoxazole and trimethoprim resistance have been found in Shigella spp. which are divided into four serogroups; S. sonnei, S. flexneri, S. boydii, and S. dysenteriae. Each serogroup is divided into several serotypes, except for S. sonnei. Hartman, et al. reported that S. sonnei has only one serotype; therefore, tracing clonal relationship is required for the identification of distinguishing epidemiological correlations. Furthermore, it has been suggested that one of the drug-resistance genes, tet (A)-1, is clonally transmitted in S. sonnei, indicating that drug-resistant strains may be traced by molecular epidemiology.

In this study, we apply a previously reported method to clarify whether a multidrug-resistant Shigella sp. colonizes in a south Asian country.

**Materials and Methods**

1. Isolation and identification of Shigella spp.

Stool samples were collected from overseas travelers who reported a history of diarrhea at the Kansai Airport Quarantine Station (KAQS) between 2001 and 2005. In this study, samples were examined by direct plating on SS agar (Eiken Chemical Co., Ltd., Tokyo, Japan) and DHL agar (Eiken Chemical Co.) plates at 37°C for 18 hr. Shigella-like colonies were screened using triple sugar iron agar (TSI; Eiken Chemical Co.) and lysine-indole-motility medium (LIM; Eiken Chemical Co.). Suspected isolates were examined by serotype testing using specific Shigella-O-antisera (Denka Seiken Co., Ltd., Tokyo, Japan). The serotyped strains were identified by biochemical tests, such as amino acid and sugar assimilation tests.

2. Antimicrobial susceptibility testing

After isolation, all isolates were examined by the disk diffusion susceptibility test for 12 antibiotics, namely, ampicillin, piperacillin, kanamycin, gentamicin, tetracycline, minocycline, chloramphenicol, fosfomycin, sulfamethoxazole-trimethoprim, nalidixic acid, ofloxacin, and levofloxacin. In brief, an isolate was cultured in tryptic soy broth, resuspended in saline at McFarland 0.5, and plated on a Mueller-Hinton agar plate (Eiken Chemical Co.). Antimicrobial agent-impregnated disks (Nippon Becton Dickinson Co., Ltd. Fukushima, Japan) were placed on the surface of the plate, the plate was cultured at 35°C for 18 hr, and the inhibition zone was measured on a transilluminator.

3. Pulsed-field gel electrophoresis (PFGE)

Agarose plugs containing DNA for PFGE were prepared using CHEF Bacterial Genomic DNA Plug kits (Bio-Rad Laboratories, Hercules, USA) according to the manufacturer’s instructions, with some modifications. In brief, bacterial cells were grown with agitation at 37°C in heart infusion broth (Difco, Detroit, USA) to a density of McFarland 2. Bacterial cells were then plugged into agarose blocks, and bacterial genomic DNA was extracted with the lysozyme and proteinase K solution supplied with the kits. Bacterial DNA in the agarose plugs was then digested with the Xba I and Sfi I restriction enzymes (TaKaRa Shuzo Co., Ltd., Otsu, Japan) at 37°C and 50°C overnight, respectively.

Electrophoresis was carried out by the contour-clamped homogeneous electric field method using a CHEF-DR II system (Bio-Rad Laboratories), at 200 V with a pulse time of 5-8sec at 14°C for 20 hr for the Xba I-digested fragment in 1% agarose gel (Pulsed Field Certified Agarose; Bio-Rad Laboratories) using 0.5× TBE buffer (pH 8.3) containing 44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA, and at 200 V with a pulse time of 5-35sec at the same temperature for 30 hr for the Sfi I-digested fragment in 1.5% agarose gel. The gels were stained with the SYBR green nucleic acid gel stain (BMA, Rockland, USA) for 30 min and photographed under a UV transilluminator (302 nm). DNA fragment patterns were visually analyzed and epidemiological relatedness was determined on the basis of the criteria for bacterial strain typing established by Tenover et al.
Results

During the period from 2001-2005, a total of 28,550 stool samples, ranging from 3,168 to 7,200 (average, 5,710) specimens/year were examined. From some of these samples, 412 Shigella spp., ranging from 53 to 106 (average, 82.4) isolates/year were isolated. Among the isolates, 327 were identified to be S. sonnei, ranging from 40 to 85 (average, 65.4) isolates/year, indicating that almost 80% of the Shigella isolates isolated at the KAQS were S. sonnei (Table 1). The most frequent country of origin of the S. sonnei isolates was India, followed in the second place by Indonesia and Cambodia, and in third place by Thailand and Viet Nam during 2001-2005 (Table 2). Because an epidemiological study requires a larger number of isolates for analysis of the long-term colonization of a strain, we focused on the S. sonnei isolates originating from India in 2001, 2002 and 2005.

To determine the antibiotic resistance pattern, the antibiotic sensitivity test was performed on these isolates from India. Unexpectedly, all of the isolates collected in 2001, 2002 and 2005 exhibited antimicrobial resistance (Table 3). The most frequent pattern of resistance was resistance to tetracycline, sulfamethoxazole-trimethoprim and nalidixic acid. The resistance patterns of resistance to tetracycline and sulfamethoxazole-trimethoprim, and to nalidixic acid alone were not frequent.

In the PFGE pattern analysis, we started typing in 2002, in which year we obtained the smallest number of S. sonnei isolates. When the PFGE band patterns were indistinguishable, the isolates were considered to be identical; otherwise, they were considered to be different. Two different patterns were detected for the isolates obtained in 2002 by Xba I digestion (Fig. 1a), which were tentatively designated as the X1 (lanes 1, 3, 4, and 5) and X2 (lane 2) types. To determine whether the same X1 or X2 type existed in the isolates of 2001, the PFGE patterns of the isolates were examined by the same method. The PFGE pattern of 15 out of the 23 isolates was the X1 type and that of the others was neither the X1 nor the X2 type.

To confirm whether all the isolates of the X1 type collected in 2002 were identical, PFGE pattern analysis with Sfi I digestion was carried out (Fig. 1b). Three different patterns were detected and tentatively designated as the S1 (lanes 1 and 2), S2 (lane 3) and S3 (lane 4) types. Further PFGE analysis with Sfi I digestion was performed on the X1-type isolates obtained in 2001. The PFGE patterns of 13 out of the 15 isolates were of the S1 type and none were of the S2 or the S3 type (Table 4).

| Table 1 | Isolation of Shigella spp. from overseas travelers with diarrhea (2001 ~ 2005) |
|---|---|---|
| **Year** | **No. of fecal specimens** | **No. of isolates** |
| | Shigella spp. | Shigella sonnei |
| 2001 | 6,891 | 106 | 85 |
| 2002 | 5,080 | 65 | 52 |
| 2003 | 3,168 | 53 | 40 |
| 2004 | 6,211 | 102 | 83 |
| 2005 | 7,200 | 86 | 67 |
| **Total** | 28,550 | 412 | 327 |

In regard to X1-S1 type isolates, two strains were detected in February, four in March, one each from April to June, three in August, and one in December.
Multidrug-resistant *S. sonnei* colonizing in India

**Fig. 1a** PFGE profiles of *S. sonnei* strains isolated in 2002 using XbaI. Four of the five strains were considered to be identical, while the strain isolated on the 7th of May was different from the others. Two different PFGE patterns were tentatively designated as the X1 type (Lanes 1, 3-5) and the X2 type (Lane 2).

**Fig. 1b** PFGE profiles of *S. sonnei* strains of the X1 type isolated in 2002 using SfiI. Three different PFGE patterns were detected and tentatively designated as the S1 type (Lanes 1-2), S2 type (Lane 3) and S3 type (Lane 4).

![PFGE profiles of S. sonnei strains isolated in 2002 using XbaI.](image)

2001. The same type of isolates were detected in April and May, 2002, and in January (two strains), February (one strain), March (two strains), May (two strains), June (one strain), August (two strains), and September (two strains), 2005. Therefore, the same type of multidrug-resistant *S. sonnei* is considered to be colonizing in India.

**Table 4** Results of PFGE analysis

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>No. of strains examined</th>
<th>No. of strains for X1-S1 type</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>2002</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2005</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

**Discussion**

The majority of the recent cases of shigellosis have been caused by *S. sonnei, S. flexneri* 2a, and *S. dysenteriae* type 13. *S. sonnei* has been isolated frequently in industrialized countries, and *S. flexneri* in developing countries13. On the basis of the data from the Infectious Agents Surveillance Report (IASR; issued by Infectious Disease Surveillance Center, National Institute for Infectious Diseases, Japan), endemic cases of shigellosis in Japan are caused mainly by *S. sonnei*. Over the last 25 years, *S. sonnei* has been the most frequently isolated bacterium in QQSs located at airports in the Kansai area. This may indicate that some of these cases of endemic shigellosis may be caused by imported *S. sonnei*, and that epidemiological analysis of the bacterium is important in Japan.

The most frequent country of origin of these isolates was India, and all of the isolates were found to be multidrug-resistant in this study as well as in a previous study14. Sur et al.15 reported that outbreaks of shigello-
sis sometimes occur in India, and Pazhani et al.\textsuperscript{15)} indicated that clonal multidrug-resistant \textit{S. dysenteriae} type 1 strains are associated with both epidemics and sporadic cases of shigellosis in India, and concluded that multidrug-resistant strains of \textit{S. dysenteriae} type 1 are spreading from outbreak-affected areas to other regions. They further noted that the reemergence of shigellosis with epidemic potential is a cause for concern, and that this trend should be carefully monitored not only in India, but also in neighboring countries.

Almost all isolates collected at JQSs originated from passengers who had traveled to only one country, indicating that the isolates originated from that country. Taniguchi et al.\textsuperscript{3)} demonstrated that the international distribution of \textit{S. sonnei} can be inferred by monitoring isolates from passengers arriving at an international airport. Using their method, we found that a multidrug-resistant strain of \textit{S. sonnei} colonizes in India, and is being continuously exported to Japan. PFGE pattern analysis has been applied to evaluate outbreaks and for disease surveillance in a country\textsuperscript{15(16)}. Tenover et al.\textsuperscript{12)} recommended criteria for the analysis of PFGE patterns, and indicated that their criteria for strain identification are stringent and are not appropriate for studies of large populations of organisms collected over extended periods of 1 year or longer. Because specimens were collected over periods of over a year in this study, we examined our isolates by PFGE analysis using two different restriction enzymes, considering above observation by Tenover et al.\textsuperscript{12)} In this study, when PFGE patterns were identical, as determined using two enzymes, we determined that the isolates were identical.

One multidrug-resistant strain showing resistance to tetracycline, sulfamethoxazole-trimethoprim and nalidixic acid was identified to be of the X1-S1 type. This type of strain was isolated from specimens collected in 2001, 2002, as well as 2005, indicating that the strain colonizes in India. Because the affected travelers had not been treated with antibiotics, these isolates were considered to have developed resistance before causing infection.

Resistances to tetracycline, sulfamethoxazole and trimethoprim originate from plasmids carrying the \textit{tet A-D} genes\textsuperscript{6(17)(18)}, \textit{dhfr gene}\textsuperscript{8(9)(10)} and \textit{sul gene}\textsuperscript{7}, respectively. Spread of these multidrug-resistant isolates may occur by the spread of antibiotic-resistant plasmids. Nalidixic acid resistance originates from mutation of the gyrase gene of chromosomal DNA\textsuperscript{5}. The multidrug-resistant strain identified in this study also exhibited resistance to nalidixic acid, indicating the occurrence of this mutation in this bacterium.

Such mutations are also known in \textit{Shigella} spp. exhibiting resistance to the newer quinolone. Talukder et al.\textsuperscript{20)} reported that a newer quinolone-resistant strain of \textit{S. dysenteriae} is spreading in South Asia. We have found only a few strains of newer quinolone-resistant isolates at present; however, surveillance for multidrug-resistant \textit{Shigella} spp., including those resistant to the newer quinolones should be performed in JQSs.

In this study, we determined that a multidrug-resistant strain colonizes in India, a country frequently visited by Japanese travelers, but we have not been able to determine the specific region of the country from which the strain originated. To identify the specific, if any, place, more information needs to be obtained from passengers with diarrhea or collaboration with surveillance institutes in India.

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日本の検疫所における遠隔疫学調査による多剤耐性赤痢菌株のインド国内における定着の証明

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我々は、検疫所で分離された菌株を解析することで海外の菌株の異同がわかるという方法を報告したが、その方法を用い多剤耐性赤痢菌が南アジアに定着しているか否かを明らかにした。関西空港検疫所において外国を旅行し下痢を罹患した者を対象に便検査を行った。2001年から2005年まで年間50〜100例赤痢菌が分離され、Shigella sonnei(S. sonnei)が約80%を占めていた。南米国別ではインドが最多であった。分離されたS. sonneiのうちインド由来株を対象として2001年、2002年、および2005年分離株について抗薬感受性試験を行ったところ、すべての菌株は薬剤耐性を示し、テトラサイクリン、スルファメトキサゾール・トリメプロミド合剤、ナリシク酸の3剤耐性株も最多2001年では25株中23株、2002年では13株中5株、2005年では19株中16株であった。次にこれらの多剤耐性株についてパルフィールドゲル電気泳動法（PFGE）による遺伝子解析を行ったところ、2001年分離株と2002年分離株の中に同じPFGEパターンを示すものが存在し、さらに2005年分離株においても同一のPFGEパターンを示す菌株が分離されていた。以上の結果から耐性赤痢菌が年をこえてインドに定着している可能性が考えられた。