Antimicrobial Susceptibilities and Molecular Epidemiology of Salmonella enterica Serotype Enteritidis Strains Isolated in Hong Kong from 1986 to 1996

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Received 12 November 1997/Returned for modification 20 January 1998/Accepted 17 March 1998

The incidence of salmonellosis has been increasing in Hong Kong since 1989. The most common Salmonella enterica serotype isolated in 1994 was S. enteritidis. The antimicrobial susceptibilities and molecular epidemiology of 275 S. enteritidis strains isolated in this locality between 1986 and 1996 were studied. Over 99% of the isolates were susceptible to 17 of the 19 antimicrobial agents tested. One isolate harbored an autotransferring plasmid that confers resistance to tetracycline and trimethoprim-sulfamethoxazole. Another isolate harbored a mobilizable plasmid that confers resistance to ampicillin and cephalothin. This isolate was found to produce a β-lactamase with a pI of 5.2. A total of 264 isolates (96%) were found to harbor one to five plasmids, and the majority (254) harbored a 60-kb plasmid. Of these isolates, 94% contained identical 60-kb plasmids. Based on plasmid profiles, plasmid and chromosomal fingerprints, ribotypes, and randomly amplified polymorphic DNA (RAPD) patterns, 170 (62%) isolates were allocated to group 1b. About 90% of isolates had identical or similar DNA fingerprints, ribotypes, and RAPD patterns, suggesting that a predominant clone of S. enteritidis was circulating in Hong Kong during the period being studied.

Despite improved sanitation, salmonellosis remains a major public health concern in many countries. The incidence of salmonella infections has been on the increase over the past 10 years. The organism that is most often responsible for the illness is Salmonella enterica serotype Enteritidis (referred to herein as S. enteritidis) (34–36, 39).

There has been a gradual but significant increase in group D salmonella infections, particularly S. enteritidis, in Hong Kong since 1989 (50). S. enteritidis has become the most-common group D Salmonella isolate and the third-most-common Salmonella serotype from extraintestinal sources in this city.

S. enteritidis is known to cause gastroenteritis and other acute infections. There is, however, little information on its antimicrobial susceptibilities and epidemiology, which would help prevent the spread of the infections and provide data about the best choices for treatment. Our aim in the present study was to investigate the antimicrobial susceptibilities and characteristics of resistant strains and the molecular epidemiology of this organism.

Materials and Methods

Bacterial strains. Two hundred fifty-two nonduplicate isolates of S. enteritidis were obtained from stools, blood, pus, or body fluids of patients admitted to the Prince of Wales Hospital in Hong Kong, China. Twenty-three more isolates were obtained from the stools of patients attending outpatient clinics. All specimens were collected between 1986 and 1996. Ninety-two percent of the strains were isolated from patients within 3 days of their admission to the hospital. The remaining strains were isolated from patients who were hospitalized for more than 3 days and could therefore have been acquired at the hospital. Four such strains were isolated from children under 5 years old. The locations where the infections originated were not known, but there was no apparent clustering of cases.

Antimicrobial susceptibilities. Susceptibilities to 19 antimicrobial agents (ampicillin, cephalothin, cefuroxime, ceftriaxone, ceftazidime, gentamicin, tobramycin, netilmicin, amikacin, streptomycin, kanamycin, nalidixic acid, ciprofloxacin, ofloxacin, rifampin, trimethoprim-sulfamethoxazole, tetracycline, and chloramphenicol) were tested by determining the MICs by the agar dilution method (Mueller-Hinton agar; Oxoid, Basingstoke, United Kingdom) and chloramphenicol.

Plasmid profiling analysis. Crude plasmids were extracted as described by Kado and Liu (12). Plasmids were separated on 0.7% agarose gels in 0.5X TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.3]) at 100 V for 4 to 5 h (GNA200 horizontal electrophoresis system; Pharmacia LKB, Uppsala, Sweden) and visualized by staining with 0.5 mg of ethidium bromide per ml.

Plasmid fingerprinting. Plasmids of the same size were purified by the method of Kado and Liu (12) and digested with EcoRI and EcoRV (Gibco BRL, Gaithersburg, Md.). Individual plasmids in strains that harbored more than one plasmid were purified from agarose gels after electrophoretic separation (Sephaglas BandPrep kit; Pharmacia Biotech Biotechnology, Uppsala, Sweden).

Transferrability of resistance plasmids. Six isolates that were resistant to more than one antibiotic but not to nalidixic acid and rifampin or highly resistant to antibiotics were selected. The isolates were tested for the ability to transfer or mobilize antibiotic resistance (2) with rifampin-resistant Escherichia coli Jp995 and transfer factors X and Δ (a gift from B. Rowe, Colindale, United Kingdom). Donors were counterselected by 64 mg of rifampin per liter in MacConkey agar (Oxoid). Characterization of β-lactamases. β-Lactamases were extracted from ampicillin-resistant isolates by sonication, subjected to isoelectric focusing on pH 3.5 to 9.5 LKB Ampholine PA Gplates (Pharmacia LKB) on an Ultrphor unit (Pharmacia LKB), and detected by 1 mg of nitrocefin (Oxoid) per liter.

Total DNA fingerprinting. Cells grown overnight in Mueller-Hinton broth were pelleted and washed with 1 ml of TE-1 buffer (50 mM Tris-HCl, 20 mM EDTA [pH 8]). The washed cell pellet was resuspended in a solution containing 540 μl of TE-2 buffer (50 mM Tris-HCl, 10 mM EDTA [pH 8]), 12 μl of a 10-mg/ml RNase solution, 30 μl of 10% sodium dodecyl sulfate (SDS), 12 μl of a 5-mg/ml lysozyme solution, and 6 μl of a 20-mg/ml proteinase K solution and incubated overnight at 56°C. The solution was extracted once with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 16 μl of 5 M NaCl and 2 volumes of ice-cold absolute alcohol. After the mixture was left overnight at −70°C, the DNA pellet was washed with ice-cold 70% alcohol, air dried, and resuspended in 50 μl of distilled water. Ten microliters of the DNA extract (about 4 μg) was digested with 10 U of EcoRV or MluI (Gibco BRL), and
TABLE 1. Characteristics of multiple-drug-resistant *Salmonella enteritidis*

<table>
<thead>
<tr>
<th>No.</th>
<th>Yr</th>
<th>Resistance pattern</th>
<th>Resistance plasmid</th>
<th>Other plasmids</th>
<th>pl of β-lactams</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1990</td>
<td>Δ (&gt;512), CTH (32), RIF (8)</td>
<td>0</td>
<td>2.6 × 10^{-5}</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td>1991</td>
<td>1 (128), SXT (&gt;128), RIF (8)</td>
<td>1.3 × 10^{-6}</td>
<td>2.5 × 10^{-6}</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td>1991</td>
<td>SXT (64), RIF (8)</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>1991</td>
<td>CXM (16), RIF (4)</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1992</td>
<td>CXM (16), S (16), RIF (8)</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>1992</td>
<td>S (16), RIF (4)</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Abbreviations: Yr, year isolated; TF, transfer frequency; pl, β-lactamase; NA, not applicable; ND, not detected; A, ampicillin; CTH, cephalothin; CXM, cefuroxime; RIF, rifampicin; SXT, trimethoprim-sulfamethoxazole.*

1. Drugs resistance to which was transferred or mobilized are underlined; MICs are shown in parentheses.

2. Frequency of mobilization.

The digested fragments were separated on a 0.4% agarose gel for 15 h at 80 V and 9 h at 100 V, respectively (GNA200 horizontal electrophoresis system; Pharmacia LKB). The nylon membrane was prehybridized in 80 ml of hybridization solution (32) at 68°C for 2 h and then hybridized overnight at 68°C in 10 ml of hybridization solution containing the cDNA probe (20 μl). The hybridized membrane was washed twice with washing solution A (2 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate], 0.1% SDS) at room temperature for 5 min and twice with washing solution B (0.1% SSC, 0.1% SDS) at 68°C for 15 min. Hybridized fragments were detected by the digoxigenin nucleic acid detection kit (Boehringer, Mannheim, Germany). The probe was labeled with the digoxigenin DNA labeling kit (Boehringer), and avian myeloblastosis virus reverse transcriptase (20 U/ml; Finnzymes, Espoo, Finland) as described by Popovic et al. (32). The labeled probe was purified by passage through a Bio-spin 30 column (Bio-Rad Laboratories Inc., Richmond, Calif.), boiled for 10 min, cooled on ice for 5 min, and then used immediately for hybridization or stored at −20°C until use.

The nylon membrane was prehybridized in 80 ml of hybridization solution (32) at 68°C for 2 h and then hybridized overnight at 68°C in 10 ml of hybridization solution containing the cDNA probe (20 μl). The hybridized membrane was washed twice with washing solution A (2 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate], 0.1% SDS) at room temperature for 5 min and twice with washing solution B (0.1% SSC, 0.1% SDS) at 68°C for 15 min. Hybridized fragments were detected by the digoxigenin nucleic acid detection kit (Boehringer).

Random-amplified polymorphic DNA (RAPD) analysis. The 15-mer oligonucleotide (TCA GCA TAG ACC TCA) (28) was used as primer. Amplification reactions were carried out in a 25-μl solution containing PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 200 μM (each) dATP, dCTP, dGTP, and dTTP, 1.0 μM primer, 1 U of Taq polymerase (Gibco BRL), and 2.5 mM MgCl₂. Approximately 40 pg of DNA was added to the mixture, which was then overlaid with 20 μl of liquid wax (Chill-out 14; MJ Research Inc., Watertown, Mass.). Amplification was carried out in a thermal cycler (PTC-100; MJ Research) at 94°C for 7 min, followed by 45 cycles (each) at 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min. A final extension of 5 min at 72°C was performed. Amplified products were electrophoresed on 0.8% gels at 100 V for 2 h (GNA100 horizontal electrophoresis system; Pharmacia LKB).

Determination of relatedness of types. The percentages of similarity between different chromosomal fingerprints, ribotypes, and RAPD types were calculated by the unweighted pair group method with arithmetic averages (38).

RESULTS

A total of 316 strains of *S. enteritidis* were isolated from human stool and extraintestinal specimens gathered from the Prince of Wales Hospital between 1986 and 1995. These represented 11% of all gastroenteric salmonellae isolated (2,909). Most of the isolates were from stools; only 16% were from blood, pus, or body fluids. Twenty-eight percent of the isolates were from patients younger than 5 years, and 37% were from patients aged between 20 and 30. The male-to-female ratio was 1:1.13. Most of the isolates (79%) were from samples gathered during the hotter months of the year (May to November).

All *S. enteritidis* isolates tested (275) were susceptible to the expanded-spectrum cephalosporins (ceftriaxone, cefotaxime, and ceftazidime; MIC range, 0.03 to 0.5 mg/liter), the 4-quinolones (ciprofloxacin and ofloxacin; MIC range, 0.0075 to 1 mg/liter), and chloramphenicol (MIC range, 1 to 8 mg/liter); all isolates were resistant to 1 mg of rifampin per liter. One isolate was resistant to ampicillin (MIC = 512 mg/liter) and cephalothin (MIC = 32 mg/liter), and two isolates were resistant to cefuroxime (MIC = 16 mg/liter). One isolate was resistant to the aminoglycoside tobramycin, and two isolates were resistant to streptomycin, but the MICs were low, 8 to 16 mg/liter. One isolate was resistant to 4 mg of tetracycline per liter, two isolates were resistant to 8 mg of nalidixic acid per liter, and two isolates were resistant to 32 mg of trimethoprim-sulfamethoxazole per liter. One isolate showed resistance to all the above antimicrobial agents.

**β-Lactamase produced by the isolate resistant to ampicillin (MIC > 512 mg/liter) and cephalothin (MIC = 32 mg/liter) had a pl of 5.2.** The two isolates that were susceptible to 16 mg of cefuroxime per liter did not produce detectable amounts of β-lactamases. Six isolates that were resistant to more than one antibiotic were tested for the transferability of their resistance (Table 1). These isolates were from samples collected from inpatients. An isolate which was resistant to tetracycline, trimethoprim-sulfamethoxazole, and rifampin transferred its resistance to an *E. coli* recipient at frequencies of 1.3 × 10⁻⁶ at 28°C and 2.5 × 10⁻⁶ at 37°C. This isolate harbored a 105-kb plasmid and a 60-kb plasmid. The tetracycline and trimethoprim-sulfamethoxazole resistance was located on the 105-kb plasmid, since only this plasmid was found in transconjugants resistant to tetracycline and trimethoprim-sulfamethoxazole. Another isolate that was resistant to ampicillin, cephalothin, and rifampin was found to have its ampicillin and cephalothin resistance mobilized by the Δ factor at a frequency of 2.6 × 10⁻⁵ at 37°C. As only a single 5.2-kb plasmid (other than the Δ transfer factor) was present in ampicillin- and cephalothin-resistant transconjugants, the ampicillin and cephalothin resistance was attributed to this plasmid. Other isolates which contained one, two, or three plasmids did not transfer their resistances or could not have their resistances mobilized by transfer factors, as resistant transconjugants could not be obtained.

The number of isolates harboring up to five plasmids with sizes ranging from 1.7 to 105 kb was 264. These isolates were allocated to 21 plasmid profiles (PP1 to PP21). The majority of isolates (226) harbored only one plasmid. Of these, 97% (219) harbored a 60-kb plasmid. Eight percent of isolates harbored two plasmids, and 3% harbored four or five plasmids. Only two isolates harbored three plasmids. The next-most-common large plasmid was 78 kb in size (harbored by eight isolates). Plasmids of other sizes were less common.

The 60- and 78-kb plasmids were digested with *EcoRI* and...
were one of these types. Most of the isolates (87%) were found of isolates, respectively. The patterns of the remaining types differed from DM1 by one fragment and were seen in 3 and 2% of the isolates were of type DM1. Types DM2 and DM3 differed from a single band, was seen in 93% of isolates. Ninety-two percent of isolates were of type DM1. Types DM2 and DM3 differed from DM1 by one fragment and were seen in 3 and 2% of isolates, respectively. The patterns of the remaining types (DM4 to DM8) were less alike; between one and four isolates were one of these types. Most of the isolates (87%) were found to have the pattern DV1-DM1. Only 5, 3, and 2% of isolates were found to have patterns DV2-DM1, DV1-DM2, and DV1-DM3, respectively. Five other patterns contained one to four isolates each.

The isolates were allocated to 15 ribotypes (R1 to R15) by PvuII restriction. The patterns were similar, differing only in one to three fragments (Fig. 3). The number of fragments which contained rRNA genes was 13 to 15, and their sizes ranged from 1.8 to 14.0 kb. R1 was the most-frequent type (91% of isolates). Only one to four isolates were found in each of the remaining ribotypes.

Six different RAPD types (PCR1 to PCR6), each with 2 to 11 fragments ranging from 0.5 to 4.0 kb, were observed among the 275 isolates. Ninety-five percent of isolates had pattern PCR1; one to five isolates were found in each of the remaining RAPD types.

Based on the pattern of plasmid profiles, plasmid and chromosomal fingerprints, ribotypes, and RAPD types, all isolates could be divided into 28 groups (1 to 28; Table 2). Isolates of the same DNA fingerprints, ribotypes, and RAPD types belonged to the same group. Each group was further subdivided according to plasmid profiles and chromosomal fingerprints. These subgroups were assigned alphabetical suffixes to the main group to indicate relatedness. There were 20 subgroups within group 1 (1a to 1t), 4 in group 2 (2a to 2d), and 3 in group 3 (3a to 3c).

Most of the isolates belonged to group 1b (62%) and were isolated from specimens obtained from inpatients and outpatients between 1987 and 1996. Isolates of groups 2, 3, and 4 (9%) differed only in plasmid and DNA fingerprints, while those of groups 5 to 28 had more varied molecular patterns.

**DISCUSSION**

Most of our *S. enteritidis* isolates were susceptible to the antibiotics tested. All were susceptible to the expanded-spectrum cephalosporins, the fluoroquinolones, and chloramphenicol, and more than 99% were susceptible to ampicillin, cephalothin, cefuroxime, aminoglycosides, nalidixic acid, tetracycline, and trimethoprim-sulfamethoxazole. The proportion of resistant strains appears to have remained unchanged over the study period. Our findings confirm those of earlier studies indicating that most strains were susceptible to a wide range of antimicrobial agents (26, 35, 37, 47).

Ampicillin resistance in *S. enteritidis* was usually due to TEM-type β-lactamases encoded by genes on a 34-, 60-, or 100-MDa plasmid (10, 43). Our only ampicillin-resistant isolate, which was also resistant to cephalothin, produced a β-lactamase of pI 5.2. The resistance was encoded on a 5.2-kb plasmid. Five β-lactamases with identical pIs (5.2) have been reported. These are HMS-1, TEM-30, TEM-31, TEM-35, and TEM-36 (22, 44, 52). Further studies on substrate profile, inhibition profile, and molecular mass may reveal which β-lactamase was produced by this isolate.

Plasmid-mediated resistance to tetracycline and trimethoprim-sulfamethoxazole or ampicillin and cephalothin was expressed in two isolates. Antibiotic resistance in other isolates was usually low and probably encoded by genes on the chromosome.

Previous studies showed that most *S. enteritidis* strains contained plasmids of up to 140 MDa in size (26, 37, 39) and that more than 70% of *S. enteritidis* strains harbored a serotype-specific plasmid of 35 to 40 MDa (11). Our findings, indicating that 96% of isolates harbored plasmids, were similar. Twenty-one plasmid profiles were identified. Profiles showing the 60-kb (36-MDa) plasmid were seen in 80% of isolates. The

**FIG. 1.** EcoRV restriction patterns of *Salmonella enteritidis* plasmids. Lanes 2 to 10, PV1 to PV9 of the 60-kb plasmid; lanes 12 to 14, PV10 to PV12 of the 78-kb plasmid; lanes 1, 11, and 15, HinfIII digest of λ DNA (Gibco BRL) as molecular size marker.
EcoRI or EcoRV restriction patterns of the 60-kb plasmid in 94% of isolates were identical, and those of the remaining isolates were similar. The only other large and commonly seen plasmid (78 kb) was found in eight isolates. This plasmid exhibited three different fingerprints.

Because of the relative instability of extrachromosomal elements in a bacterial cell, total DNA was used to elucidate the epidemiology of S. enteritidis. DNA fingerprinting, ribotyping, and RAPD analysis showed that more than 90% of isolates had the same patterns, regardless of where and when they were isolated. When the patterns obtained by all the methods used in this study were combined, 62% of the isolates were found to be identical and to belong to group 1b. Isolates in other subgroups of group 1 and those in groups 2 to 4 were probably variants of isolates of group 1b. They differed from those of group 1b only in plasmid content or DNA fingerprints (DV1-DM2, DV2-DM1, or DV1-DM3). DNA fingerprints DV1 and DV2 and DM1, DM2, and DM3 were similar. The isolates in these groups (groups 1 to 4) were from specimens collected from inpatients and outpatients between 1987 and 1996. It is therefore possible that a predominant clone and a few related clones of S. enteritidis were circulating in Hong Kong during this period. Since a strain isolated in 1986 belonged to a very different genotype, the upsurge of the incidence of S. enteritidis in 1989 may have been due to the spread of strains isolated in 1987.

Studies from other parts of the world support the hypothesis that there is a clonal relationship between different S. enteri-
**SALMONELLA ENTERITIDIS FROM HONG KONG**

**TABLE 2. Pattern combination of plasmid profiles, plasmid and chromosomal fingerprints, ribotypes, and RAPD types**

<table>
<thead>
<tr>
<th>Pattern</th>
<th>PP</th>
<th>PV</th>
<th>PI/PV</th>
<th>DV</th>
<th>DM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Nil</td>
<td>DV1</td>
<td>DM1</td>
<td>R1</td>
<td>PCR1</td>
<td>27 (3)</td>
</tr>
<tr>
<td>1b</td>
<td>PP1</td>
<td>PI1/PV1</td>
<td>DV1</td>
<td>DM1</td>
<td>R1</td>
<td>PCR1</td>
</tr>
<tr>
<td>1c</td>
<td>PP1</td>
<td>PI2/PV2</td>
<td>DV1</td>
<td>DM1</td>
<td>R1</td>
<td>PCR1</td>
</tr>
<tr>
<td>1d-1t</td>
<td>Various</td>
<td>Various</td>
<td>DV1</td>
<td>DM1</td>
<td>R1</td>
<td>PCR1</td>
</tr>
<tr>
<td>2a-2d</td>
<td>Various</td>
<td>Various</td>
<td>DV1</td>
<td>DM2</td>
<td>R1</td>
<td>PCR1</td>
</tr>
<tr>
<td>3a-3c</td>
<td>Various</td>
<td>Various</td>
<td>DV2</td>
<td>DM1</td>
<td>R1</td>
<td>PCR1</td>
</tr>
<tr>
<td>4</td>
<td>PP1</td>
<td>PI1/PV1</td>
<td>DV1</td>
<td>DM3</td>
<td>R1</td>
<td>PCR1</td>
</tr>
<tr>
<td>5-28</td>
<td>Various</td>
<td>Various</td>
<td>DV1-DV4</td>
<td>DM1-DM8</td>
<td>R1-R15</td>
<td>PCR-PCR6</td>
</tr>
</tbody>
</table>

**Abbreviations:** PP, plasmid profile; PV, plasmid fingerprint; PI, chromosomal fingerprint by EcoRV restriction; DM, chromosomal fingerprint by MluI restriction; R, ribotype; PCR, RAPD pattern; OP, outpatients.

**FIG. 3. PvuII ribotypes of Salmonella enteritidis.** Lanes 2 to 16, R1 to R15; digoxigenin-labelled DNA molecular size markers III (21, 5.2, 5.0, 4.3, 3.5, 2.0, 1.9, and 1.6 kb) and VII (8.0, 7.1, 6.0, 4.8, 3.5, 2.7, 1.9, 1.8, and 1.5 kb) (both from Boehringer) are used in lanes 1 and 17 and lane 18, respectively.
detecting genomic diversity among different organisms (1, 14, 20, 23, 51) and has been useful for studying the epidemiology of S. enteritidis (9, 19). However, different primers have to be tested first, and conditions of PCR must be optimized for each organism studied (8). Suitable primers should have a G+C content greater than 40% (49). The discriminatory power of this method can be increased by using two primers (24, 48).

The present study showed that a single method cannot be relied upon for discriminating between S. enteritidis strains. Isolates with the same DNA fingerprints and PCR types (groups 9 to 21) can belong to different ribotypes. Those with the same DNA fingerprints and ribotyping patterns (groups 25 to 28) can exhibit different PCR patterns. It is therefore necessary to combine the results of different molecular typing methods for reporting the epidemiology of S. enteritidis.


