Full Length Research Paper

Clonal expansion and genetic diversity of nalidixic acid-resistant Salmonella enterica serotype Paratyphi A

Shukun Wang¹, Yunbo Yao¹, Congjia Chu², Desheng Shan², Biao Kan³, Baowei Diao³ and Xianhua Wu⁴*

¹Centre for Disease Control and prevention of Yuxi city, Yuxi, Yunnan province, China.
²People’s Hospital of Yuxi City, Yuxi, Yunnan province, China.
³National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping, Beijing, China.
⁴Yuxi Normal University, Yuxi, Yunnan province, China.

Accepted 9 December, 2011

The aim of this study was to understand the clonal expansion and genetic diversity of nalidixic acid-resistant (NAR) Salmonella enterica serotype Paratyphi A (SPA) from Yuxi city, China. Antimicrobial susceptibility testing was performed with 3980 SPA isolates from Yuxi city of China between 1999 and 2007. The incidence of resistance to nalidixic acid (NA) of 1999 and 2000 were 25.0 and 82.2%, respectively. More than 95.0% of the isolates obtained from 2001 to 2007 were resistant to NA. Amplification by PCR and sequencing of the genes within the quinolone resistance-determining region with subsets of 15 NAR strains revealed that the mechanisms of NA resistance resulted from single point mutations in the gyrA gene leading to the Ser83Phe substitution. Subtyping of 120 NAR isolates from seven counties and one Nalidixic acid-susceptible (NAS) isolate were studied using pulsed-field gel electrophoresis (PFGE) analysis, followed by digestion of chromosomal DNA with restriction endonucleases SpeI and XbaI. PFGE patterns were analyzed by cluster analysis. SpeI and XbaI digestion of 121 isolates generated five and four different PFGE patterns with predominance of the SpeI01 and SpeI02 (or the XbaI01) epidemic patterns, respectively. The incidence of resistance to NA of the isolates increased during the study period. NAS isolates predominated in 1999 but was replaced by NAR isolates after 2000. PFGE patterns SpeI01 and SpeI02 (or XbaI01), the main clones of the epidemics, are highly prevalent in Yuxi.

Key words: Paratyphoid fever, Salmonella enterica serotype Paratyphi, antimicrobial susceptibility, resistance to nalidixic acid, PFGE, clonal expansion.

INTRODUCTION

Enteric fever remains a serious public-health problem in many regions of the world (Bhan et al., 2005; Crump et al., 2004). The aetiologic agents are Salmonella enterica serotype Typhi, and Salmonella enterica serotype Paratyphi A, B, and C (Parry et al., 2002; Brenner et al., 2000). Although paratyphoid fever A (PA) is less severe than typhoid fever (Everest et al., 2001; Bhan et al., 2005; Parry et al., 2002), recent disease activities in Yuxi of China suggests that Salmonella enterica serotype Paratyphi A (SPA) could increase in importance and represents a challenge to human in the future (Fanlin et al., 2005).

Yuxi city (a 15 285-km² area with 2, 095, 532 registered inhabitants) is the most severe endemic area of PA in China (Fanlin et al., 2005). The incidence of PA abruptly increased in and after 1999. Blood culture results obtained between January 1999 and December 2007 were reviewed for patients admitted with suspected PA to
hospital of Yuxi City. SPA was isolated from 3980 of 12,285 patients by aerobic and anaerobic culture bottles (AER/ANA, bioMérieux, France). The isolates of SPA from Yuxi, which cause 42% (8/19) and 97% (73/75) of culture-proven enteric fever cases in 1999 and 2000, have been increasing. The ratios of culture-proven PA to typhoid fever cases were 8:11, 73:2, 926:2, 484:6, 259:6, 751:1, 419:1, 608:1, and 452:0 per year from 2001 to 2007, respectively. The incidences of PA and typhoid fever, in Hongta area (a 1,004-km² area with 420,000 registered inhabitants) of Yuxi, were 358, 125, 108, 290, 132, 329, and 191 cases per 100,000 population per year from 2001 to 2007, respectively. However, the genetic diversity of SPA epidemic in Yuxi remains unclear. Therefore it is useful to clarify genetic patterns of SPA isolated in Yuxi in order to provide better advices to control and prevent PA.

To develop effective control strategies, it is vital to characterize accurately the extent of genetic and phenotypic variation present in the pathogen population. The technique of pulsed-field gel electrophoresis (PFGE), in particular, has been the most widely used method for molecular epidemiological investigations of infections caused by a range of bacterial pathogens, and is currently the standard method for molecular typing of Salmonella spp. (Sandt et al., 2006; Saida et al., 2007; Goh et al., 2002). Definition of the antimicrobial susceptibility and genetic diversity of SPA isolates may be helpful in the management of individual cases and outbreaks (Perron et al., 2007; Le et al., 2007; Bhan et al., 2005; Parry et al., 2002). It is useful to determine whether the endemic, epidemic PA found throughout Yuxi from 1999 to 2007 was due mainly to multiple SPA clones or a single one, and understand the mode of spread of SPA, in order to implement rational strategies and suitable measures for the prevention and control of PA in the field of public health.

MATERIALS AND METHODS

Bacterial strains

A total of 3980 isolates of SPA were obtained from different individuals admitted with PA to hospital of Yuxi City between January, 1999 and December, 2007 (Table 1). All organisms isolated following subculture were identified to the serotype level by agglutination tests with Salmonella O2- and H-specific antiserum (Biological products institute of Ministry of Public Health, Lanzhou, China) and standard biochemical tests (Automatic Microorganism Identification System VITEK 32 CNI+ or API 20E, bioMérieux, France) (Ekdahl et al., 2005).

A total of 15 sporadic isolates (one per patient) of SPA from Hongta (5 isolates), Chengjiang (3 isolates), Xining (3 isolates), Eshan (1 isolates), Tonghai (1 isolates), Huaning (1 isolates), and Jiangchuan (1 isolates) were selected from blood cultures in hospitals located in the seven regions of Yuxi and epidemiologically independent. The isolates were used for the analysis on the mechanisms of nalidixic acid (NA) resistance. A total of 121 sporadic isolates (one per patient) of SPA from Hongta (80 isolates), Chengjiang (12 isolates), Xining (8 isolates), Eshan (7 isolates), Tonghai (6 isolates), Huaning (6 isolates), and Jiangchuan (2 isolates) were collected from April to October 2007. These isolates were selected from blood cultures in hospitals located in the seven regions of Yuxi and epidemiologically independent. The isolates were used for PFGE analysis.

One strain of Escherichia coli ATCC 25922 (with known MICs) was used as control for potency of antibiotics. The molecular size marker used for all PFGE gels was XbaI-digested DNA from Salmonella enterica serovar Braenderup H9812 (American Type Culture Collection catalog no. BAA-664). The universal size standard was used by all PulseNet laboratories.

Antimicrobial susceptibility testing

The isolates were tested for susceptibility to antimicrobials by the controlled Kirby-Bauer disc diffusion technique on Muller-Hinton agar (Oxoid, Basingstoke, United Kingdom) plates (CLSI, 2007). The antibiotic disks (Oxoid Limited, Hampshire, England; the disk content is indicated in parentheses) contained ampicillin (Am, 10 µg), amoxycillin (25 µg), cefotaxime (30 µg), cefotaxime (30 µg), cefotaxime (30 µg), imipenem (10 µg), piperacillin (25 µg), andampicillin (5 µg), levofloxacin (5 µg), lomefloxacin (10 µg), norfloxacin (10 µg), nalidixic acid (40 µg), sulphamethoxazole (25 µg), tetracycline (30 µg), and chloramphenicol (30 µg).

For quality control of the culture media and antimicrobial disks, Escherichia coli ATCC 25922 were tested under the same conditions. The stability of the antimicrobial resistance of a number of the isolates was determined by subculturing representative isolates each day for 3 weeks (The measurements were repeated 3 times). At the end of each week the isolates were retested against the same panel of antimicrobial agents.

PCR

15 isolates were prepared for PCR (Kariuki et al., 2004; Butt et al., 2003) by individually suspending the bacterial cells, grown on tryptic soy agar at 37°C, into a 50 µl cell lysis buffer (200 ng/ml protease K), and incubated for 1.0 h at 55°C in a shaking water bath. Total DNA was prepared by boiling SPA for 10 min, followed by centrifugation at 15000 rpm for 2 min to obtain the supernatant. PCR was performed with the four primer pairs (Table 2). Reaction conditions consisted of 5 µl DNA supernatant, 1µl of each primer, 0.5 µl of 25 mM deoxynucleoside triphosphate, 30.5 µl ultra-pure water in a 10 µl buffer composed of 5×PCR buffer, and 2µl Taq polymerase (2 U/µl) mixture in a final volume of 50 µl. Amplification conditions consisted of 40 cycles of 93°C for 5 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 7 min. To determine whether a mutation had occurred at the gyrA, gyrB, parC and parE genes, 2 µl of purified PCR product each was digested with 5 U of HindII at 37°C for 2 h. PCR products were resolved by electrophoresis on 1.5% (w/v) SeaKem Gold agarose gels at 120 V for 1 h. The gels were stained with ethidium bromide (0.5 µg/ml; Sigma) for 25-30 min, destained in distilled water for 60-90 min, photographed under uv illumination, and recorded on a Gel Doc system (Bio-Rad Laboratories, Inc., Hercules, CA).

Determination of mutations within gyrA, gyrB, parC, and parE

Mutations in gyrA, gyrB, parC, and parE were determined by using sequencing of the gyrA, gyrB, parC, and parE amplification (Kariuki et al., 2004). For sequence analysis, the genes were amplified by PCR from the extracted genomic DNA of the isolates using the primers (Table 2, Lark Technologies, Inc., Essex, United Kingdom).
Comparison of Standard nucleotide sequencing and the DNA sequencing was analyzed by using a commercial software (Lasergene: DNASTAR, Inc., Madison, Wis.) and used to compare the Standard nucleotide sequencing CP000026 from BLAST in Genbank and RefSeq used to cDNA sequencing that was prepared for the susceptibility testing is Escherichia coli ATCC 2592.

The DNA sequencing was analyzed by using a commercial software (Lasergene: DNASTAR, Inc., Madison, Wis.) and used to compare the Standard nucleotide sequencing CP000026 from BLAST in Genbank and RefSeq used to cDNA sequencing that was prepared for the susceptibility testing is Escherichia coli ATCC 2592.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>8</td>
<td>20.0</td>
<td>73</td>
<td>12.3</td>
<td>926</td>
<td>8.4</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>8</td>
<td>0.0</td>
<td>73</td>
<td>13.7</td>
<td>926</td>
<td>9.0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>8</td>
<td>0.0</td>
<td>73</td>
<td>0.0</td>
<td>926</td>
<td>0.0</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>8</td>
<td>0.0</td>
<td>73</td>
<td>0.0</td>
<td>926</td>
<td>0.0</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>8</td>
<td>0.0</td>
<td>73</td>
<td>2.5</td>
<td>926</td>
<td>1.9</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>8</td>
<td>25.0</td>
<td>73</td>
<td>82.2</td>
<td>926</td>
<td>95.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>106</td>
<td>66.0</td>
<td>196</td>
<td>69.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>106</td>
<td>0.0</td>
<td>196</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>106</td>
<td>1.9</td>
<td>196</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td>106</td>
<td>96.2</td>
<td>196</td>
<td>97.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>8</td>
<td>0.0</td>
<td>73</td>
<td>0.0</td>
<td>926</td>
<td>0.2</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>106</td>
<td>0.0</td>
<td>196</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>8</td>
<td>0.0</td>
<td>73</td>
<td>5.5</td>
<td>0.5</td>
<td>743</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>106</td>
<td>0.0</td>
<td>196</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Antibiotics and the amounts in disks were as follows: ciprofloxacin, levofloxacin, 5 µg each; ampicillin, imipenem, norfloxacin, lomefloxacin, 10 µg each; amoxicillin, sulphamethoxazole, 25 µg each; cefotaxime, cefoxitin, ceftazidime, nalidixic acid, tetracycline, chloramphenicol, 30 µg each.

The quality control strains that were used for the susceptibility testing is Escherichia coli ATCC 2592.

The procedure was performed according to the PulseNet website (http://www.cdc.gov/pulsenet/protocols.htm) (Gaul et al., 2007; Sandt et al., 2006). Restriction endonucleases XbaI and SpeI (TaKaRa Biotechnology Co, Ltd, Dalian, China) were used. A Gel Doc 2000 equipped with Quantity one software (Bio-Rad, Hercules, CA) was used for image capture and conversion of gel images to the TIF file format. The file images were processed by BioNumerics software version 4.6 (Applied Maths, BVBA, Kortrijk, Belgium).

121 isolates of SPA from were prepared for PFGE by individually suspending the bacterial cells, grown on tryptic soy agar at 37°C, into a cell suspension buffer (100 mmol l⁻¹ Tris-HCL, 100 mmol l⁻¹ EDTA, pH 8.0) to a spectrometer absorbance of 0.7±0.05 at 612 nm (Optical density of 3.6 to 4.5 in bioMerieux Vetek colorimeter, France). Proteinase K (20 µl) was added to 400 µl of the suspension along with 400 µl of molten (56°C) 1% (w/v) SeaKem Gold Agar. These were mixed quickly, and approximately 400 µl was dispensed into prepared plug molds. Once solidified (10-15 min), the plugs were placed into 5 ml cell lysis buffer (50 mmol l⁻¹ Tris-HCL, 50 mmol l⁻¹ EDTA, pH 8.0, 1% Sarcosyl) and 25 µl of Proteinase K and incubated for 2.0 h at 54°C in a shaking water bath. The plugs were washed twice in ultra-pure water for 15 min in a 50°C water bath followed by four washings in Tris-EDTA (TE) buffer (10 mmol l⁻¹ Tris-HCL, 1 mmol l⁻¹ EDTA, pH 8.0). Chromosomal DNA from SPA isolates was prepared in the washed plug, and the plugs were stored in TE buffer at 4°C. For PFGE, the plugs were cut into 2 by 9 mm pieces and then digested in 156 µl of sterile water, 20 µl of bovine serum albumin, 20 µl of 10× ReActII buffer, and 4 µl of restriction endonucleases SpelI or XbaI or BinI at 37°C in a shaking water bath for 2.0 h.

**PFGE analysis**

The plugs were run in a 1% (w/v) SeaKem Gold agarose gel using a CHEF-DR III Pulsed-Field System (Bio-Rad, USA) in 0.5% Tris-borate-EDTA buffer (Sigma) at 10-14°C. The parameters were set with the initial switch time at 2.2 s, the final switch time at 63.8 s, a voltage of 6V/cm, and a duration of 19.5 h. Included on the gel were SpelI or XbaI or BinI at 37°C in a shaking water bath for 2.0 h.

The file images were processed by BioNumerics software version 4.6 (Applied Maths, BVBA, Kortrijk, Belgium).
Table 2. Primers used for PCR amplification and sequencing of genes coding for the quinolone resistance.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>GYRA</td>
<td>5′-TGTCGAGATGGCCTGAAGC-3′</td>
<td>470</td>
</tr>
<tr>
<td></td>
<td>5′-CGTTAATCAGCTCCGTACAG-3′</td>
<td></td>
</tr>
<tr>
<td>GYRB</td>
<td>5′-AAGCGCCGACGCAAGGAAAG-3′</td>
<td>566</td>
</tr>
<tr>
<td></td>
<td>5′-CCTTTACGACGGGTCATTTC-3′</td>
<td></td>
</tr>
<tr>
<td>PARC</td>
<td>5′-ATGAGCGATATGGCAGAAG-3′</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>5′-TGACGGAGCTTGACCGAAGCAG-3′</td>
<td></td>
</tr>
<tr>
<td>PARE</td>
<td>5′-GACCGAGCTGTCCTTGTGG-3′</td>
<td>493</td>
</tr>
<tr>
<td></td>
<td>5′-GCCTACGCTCGGCGTTCA-3′</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Electrophoresis pattern of PCR product from amplification of gyrA gene.

destained in distilled water for 60-90 min, photographed under UV illumination, and recorded on a gel doc system (Bio-Rad Laboratories, Inc., Hercules, CA). Isolates were considered to be genetically similar or identical if there was complete concordance of the DNA fragment profiles and were considered different if there was a difference of one or more DNA bands. All isolates within a PFGE subtype had identical bands, therefore one isolate from each PFGE subtype was randomly selected as a representative and cluster analysis was completed on the subtypes by the unweighted pair-group method with arithmetic averages (UPGMA), based on the Dice similarity index. A 0.5% optimization parameter and a 1.5% band position tolerance were used.

RESULTS

Antibiotic susceptibility testing

The 3, 980 isolates exhibit a high incidence of resistance to nalidixic acid, and the rates of 1999 and 2000 were 25.0 and 82.2%, respectively. During 1999-2000, 19 (23.5%) of 81 isolates were susceptible to nalidixic acid. The incidence of PA caused by NAR strains abruptly increased (up to 95.0% of isolates) in 2001. More than 95.0% of the isolates obtained from 2001 to 2007 were resistant to NA. There was a significance change in the resistance pattern to NA during the study period. However, all the tested isolates were susceptible to cefotaxime, ceftazidime, tetracycline, and chloramphenicol. The most tested isolates were susceptible to imipenem, sulphamethoxazole, ampicillin, amoxicillin, cefoxitin, particularly to imipenem and sulphamethoxazole. The susceptibility tests for the tested isolates showed that the most isolates were resistant to fluoroquinolones (Table 1).

Analysis of QRDR of SPA

We examined a total of 15 SPA with nalidixic acid resistance for mutations in gyrA, gyrB, parC and parE genes within the QRDR. Amplification of the gyrA, gyrB, parC and parE genes from all strains by PCR and restriction electrophoresis of PCR products revealed one band each, the findings which indicated that the strains all contain the specific genes. Nucleotide sequence analysis showed that point mutation (TCC was switched to TTC, Ser-83 to Phe) had occurred in the QRDR in the gyrA of 15 resistant isolates (Figures 1 and 2); no point mutation
Figure 2. The sequencing pattern on point mutation of gyrA gene.

Figure 3. PFGE patterns from representative strains of SPA collected from patients following digestion with SpeI. Pattern SpeI01 and SpeI02: epidemic clone in Yuxi region.

had occurred in the gyrB, parC and parE genes of 15 strains.

PFGE patterns

In the analysis of fragments produced by SpeI and XbaI digestion of genomic DNA from 121 isolates of SPA. Analysis by PFGE of restriction fragments from the SpeI- and XbaI- digestion of genomic DNA produced 5 and 4 distinct patterns (Figures 3, 4, 5 and 6), respectively. The sources and PFGE patterns of SPA isolates from different geographical regions were summed in Table 3. PFGE pattern SpeI01 and SpeI02 consisted of 37.2% (45/121)
Figure 4. Cluster analysis of the PFGE SpeI fragment pattern of SPA from representative strains of SPA collected from patients (UPGMA, Dice similarity coefficient; band tolerance, 1.5%).

Figure 5. PFGE pattern from representative strains of SPA collected from patients following digestion with XbaI. Pattern XbaI01: epidemic clone.

and 57.9% (70/121) of isolates, respectively, or pattern XbaI01 consisted of 95.1% (115/121) of isolates, pattern
Table 3. Sources and PFGE patterns of SPA isolates from Yuxi regions.

<table>
<thead>
<tr>
<th>County of origin</th>
<th>No. of strains</th>
<th>PFGE pattern SpeI</th>
<th>PFGE pattern XbaI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>01    02  03  04  05</td>
<td>01    02  03  04  05</td>
</tr>
<tr>
<td>Hongta</td>
<td>80</td>
<td>32    47  1</td>
<td>79    1</td>
</tr>
<tr>
<td>Chengjiang</td>
<td>12</td>
<td>2     6    4</td>
<td>8     4</td>
</tr>
<tr>
<td>Xinping</td>
<td>8</td>
<td>2     6    4</td>
<td>8     4</td>
</tr>
<tr>
<td>Eshan</td>
<td>7</td>
<td>3     4    2</td>
<td>7     6</td>
</tr>
<tr>
<td>Tonghai</td>
<td>6</td>
<td>3     3    6</td>
<td>6     6</td>
</tr>
<tr>
<td>Huaning</td>
<td>6</td>
<td>3     3    6</td>
<td>6     6</td>
</tr>
<tr>
<td>Jiangchuan</td>
<td>2</td>
<td>0     1    1</td>
<td>1     1</td>
</tr>
<tr>
<td>Total</td>
<td>121</td>
<td>45    70  4</td>
<td>115   4</td>
</tr>
</tbody>
</table>

SpeI03 or XbaI02 consisted of 3.3% (4/121) of isolates, both pattern SpeI04 or XbaI03 and SpeI05 or XbaI04 consisted of 0.8% of isolates. Five patterns which differed in only 1 to 3 bands were shared among the isolates indicating that these isolates were closely related. Among the various PFGE patterns identified, most isolates belonged to 1-2 predominant patterns, pattern SpeI01 and SpeI02 or pattern XbaI01, the main cause of the epidemics, is highly prevalent in humans in 7 counties of Yuxi. Surveillance of 121 SPA isolates demonstrated a surge in PFGE pattern SpeI01 and SpeI02 (or XbaI01) isolates. SpeI03 or XbaI02 (from Chengjiang), SpeI04 or XbaI03 (from Hongta), and SpeI04 or XbaI04 (from Jiangchuan) isolates tested did not match the epidemic patterns and were considered to be unrelated sporadic patterns or occurrence of macroevolution (generation-to-generation small-scale genetic changes in a population) within the epidemic patterns.

Cluster analysis of PFGE patterns

Five clusters of the 121 SPA isolates were identified by PFGE (Figures 4 and 6; Table 3). The bands on the gel have been characterized based on size in kilobase pairs. Based on the 97.8% similarity, there were 2 clusters. The 45 isolates from Hongta, Chengjiang, Xinping, Eshan, Tonghai, and Huaning were grouped into one cluster which shared pattern SpeI01. The 70 isolates from Hongta, Chengjiang, Xinping, Eshan, Tonghai, Huaning, and Jiangchuan were grouped into another cluster which shared pattern SpeI02. Based on the 96.8% similarity, there were 2 major clusters. The 115 isolates from Hongta, Chengjiang, Xinping, Eshan, Tonghai, Huaning, and
Jiangchuan were grouped into one major cluster which shared pattern SpeI01 and SpeI02 or XbaI01. The 4 isolates from Chengjiang were grouped into another cluster which shared pattern SpeI03 or XbaI02. In contrast, the 2 sporadic isolates from Hongta and Jiangchuan gave PFGE patterns (SpeI04 or XbaI03 and SpeI05 or XbaI04) which were unique and distinctly different from one another.

**Antibiotic susceptibility and PFGE pattern**

The results obtained by antibiotic susceptibility testing support the existence of distinct groups. It was noted that resistance to NA was not associated with a particular PFGE pattern. No correlation was observed between the presence/absence of the Ser83Phe substitution and any PFGE pattern. PFGE was able to subtype the NAR strains into four groups [SpeI01 and SpeI02 (or XbaI01), SpeI03 or XbaI02, SpeI04 or XbaI03]. One nalidixic acid-susceptible strain from Jiangchuan gave PFGE pattern SpeI05 or XbaI04.

**DISCUSSION**

SPA is highly prevalent and remains endemic in humans in Yuxi (Fanlin et al., 2005). Since the first report of SPA cases and NAR isolates, which occurred in Hongta in 1999 when the prevalence of the NAR phenotype was 25% (2/8), continuous surveillance has shown that the prevalence of the NAR phenotype has been rising steadily and that, at present, more than 99% of all SPA isolates from blood cultures from the main referral hospital in Yuxi are NAR. NAS isolates predominated in 1999 but was replaced by NAR isolates after 2000 (Table 1). 62 resistant strains of 83 isolates between 1999 and 2000 were NAR. 3787 resistant strains of 3899 isolates between 2001 and 2007 were NAR. After NAR SPA epidemic occurred in Hongta during 2001, the NAR strains have been isolated from all Yuxi counties, mainly in Hongta where PA remains endemic. Based on the increases in the prevalence of the NAR phenotype, it appears that NAR strains have been spreading to other parts of Yuxi and are gradually replacing the fully sensitive strain type, probably due to their survival advantage over sensitive strains or widespread use of quinolones. The emergence of NAR isolates is of great concern because these strains are associated with slow clinical responses to fluoroquinolones and treatment failures (Giraud et al., 2006; Threlfall et al., 2003; Harish et al., 2004). The observation that most of the isolates shared the same NAR phenotype is indicative of the spread of a single resistant clone of SPA. This means that the endemic, epidemic NAR strains in Yuxi was due for the most part to a single NAR bacterial clone spreading from Hongta (the original epidemic) to the other counties from 1999 to 2007.

We screened for mutations in gyrA, gyrB, parC and parE genes within the QRDR to target the reason for the high incidence of NA resistance among the isolates. The screening for mutations and sequencing of the PCR products within the gyrase gene in 15 NAR isolates reveal a point mutation (Ser-83 to Phe) in the gyrA gene within the QRDR of SPA cause the resistant to NA and reduced susceptibility to fluoroquinolones. The property of NA resistance was chromosomal-based (Giraud et al., 2006; Butt et al., 2003). It was shown that mutations in gyrA can be sufficient to cause high-level resistance to NA in Salmonella (Giraud E et al., 2006).

PFGE of SpeI- and XbaI- digested chromosomal DNA from 121 SPA isolates produced 5 and 4 distinct patterns, respectively, showing that 5 strain types are in circulation in endemic areas but epidemics are related to one or two strain types. PFGE patterns SpeI01 and SpeI02 (or XbaI01), the main clones of the epidemics, are highly prevalent in seven counties of Yuxi. SpeI03 or XbaI02, SpeI04 or XbaI03, and SpeI05 or XbaI04 isolates tested did not match the epidemic patterns and were may be unrelated sporadic clones or the microevolution within the epidemic clone (Le TAH et al., 2007). PFGE indicated the presence of just one predominant cluster XbaI01 (n=115) or two predominant clusters SpeI01 (n=45) and SpeI02 (n=70). The group XbaI01 or group SpeI01 and SpeI02, appears to be very homogeneous, with only one minor variant (profiles differed by a band of less than 100 kb) detected by PFGE of SpeI- digested chromosomal DNA. Greater diversity (profiles differed by one or more bands of > 100 kb) was observed among group XbaI02 or SpeI03, XbaI03 or SpeI04, and XbaI04 or SpeI05 isolates (which mainly correlate with sporadic cases or the microevolution). We can assume that most of the PA cases which occurred over nine years in Yuxi were due to the clonal expansion of one or two strains, depending on whether the distinction between SpeI01 and SpeI02 is reliable or not (Le et al., 2007). If the distinction is reliable, two predominant clones have coexisted in Yuxi, at least in Hongta, since 1999 and even before. If the distinction is not reliable, and taking into account a proportional part of the other strains or the occurrence of microevolution within the clone (Le TAH et al., 2007). The application of PFGE to SPA from seven people who lived in seven separate areas in Yuxi showed that the predominant isolates were virtually identical to the strain associated with infection in Hongta. 87.8% (36/41) of SPA isolates from the affected parts of Yuxi except for Hongta had PFGE patterns similar to those of isolates from Hongta, and this strain type seems to be predominant in the seven study areas examined here. It is likely that epidemics from these seven regions were caused by the same strains. In Asia in general, there have been reports describing many PFGE patterns in circulation (Goh et al., 2002). PFGE
suggested that individual outbreaks were associated with closely related strains, whereas isolates of SPA from sporadic cases were very diverse (Thong et al., 2000). A high incidence could arise from either rare mutation events with subsequent clonal expansion and dissemination or from mutation and selection events (Kilmartin et al., 2005).

A good correlation established between the heterogeneous patterns obtained from both SpeI and XbaI PFGE from 121 isolates lead us to estimate that the higher discriminatory power of XbaI PFGE displays clonal variations more than independent clones, whereas SpeI PFGE is a solid tool to separate real clones and it is useful for detecting very new microevolution within the clone. SpeI PFGE could not only recognize homogeneous clones related to outbreaks and epidemics, but also discriminate isolates from sporadic cases. These figures indicate that PFGE had high discriminatory power and so is an accurate epidemiological tool, in any case for SPA. It appears suitable to determine whether SPA isolates belong to the same clone or not. Our study was intended to provide baseline data for the application of PFGE to the routine typing of SPA isolates and to the investigation of possible common-source outbreaks of SPA. The 115 isolates that made up SpeI01 and SpeI02 (or XbaI01) were primarily isolated from patients with no apparent connection from seven counties. There was a few indication of a common-source outbreak during the period when the isolates were collected. The findings may suggest fecal-salad vegetable-fecal and person-to-person transmission of a particular strain over an extended period of time, although this interpretation must be regarded tentative, given the lack of detailed case studies (Vollaard et al., 2004). The application of the standardized PulseNet protocol to representative isolates from this collection may allow comparison with other data to determine if SPA isolates with similar PFGE patterns also occur elsewhere in the world and over time should permit us to determine if the periodic increases in the occurrence of SPA observed in Yuxi are associated with the emergence of new strains.

Continued monitoring of clonal expansion and antimicrobial resistance among SPA isolates and communication between physicians and reliable medical biology laboratories will facilitate determination of prevention and treatment policies. Prevention strategies are becoming more important in the face of increasing NA resistance. Suitable measures in the field of antibiotic administration are needed to prevent increasing levels of resistance to fluoroquinolone, in particular regarding the reuse of classical antibiotics. Recommendation for the emergency treatment of outbreaks and epidemics caused by a fully fluoroquinolone-resistant strain can be made. PFGE with SpeI and XbaI was a useful technique to differentiate SPA from different geographical regions, and can be useful for epidemiological routine investigation of PA. The outcome of our study will lead to the implementation of rational strategies and suitable measures in the field of public health in order to control and prevent PA.

ACKNOWLEDGEMENTS

This study was supported by Chinese center for disease control and prevention, People’s Republic of China. We are very grateful to the physicians and nurses from yuxi city hospital for their assistance and cooperation.

REFERENCES

Saida NB, Mhalla S, Bouzouia N, Boukadida J (2007). Genotypic analysis of Salmonella enterica serovar Typhi collected during two


