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Nalidixic acid resistance and clonal expansion of *SALMONELLA ENTERICA* serotype Paratyphi A Isolates in Yuxi city, China

W. Shukun¹, C.Congjia², Y. Yunbo¹, K. Biao³, and D. Baowei³

¹Centre for Disease Control and prevention of Yuxi city, Yuxi 653100, China. ²People's Hospital of Yuxi city, Yuxi 653100, China. ³National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, P.O. Box 5, Changping, Beijing 102206, China

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Antimicrobial susceptibility tests were performed with 4060 strains of *SALMONELLA ENTERICA* serotype Paratyphi A (SPA) isolated from patients at Yuxi City from 1999 to 2008. The incidence of resistance to nalidixic acid of the isolates increased from 12.5% in 1999 to 82.2% in 2000, 93.0% in 2001, and 100% in 2008. The frequencies of intermediary and susceptibility (Kirby-Bauer disc diffusion technique) to ciprofloxacin were 17.0% and 83.0% before 2005, 65.3% and 34.7% in 2008, respectively. A group of 166 NAR and 20 NAS isolates of SPA were typed by pulsed-field gel electrophoresis (PFGE) using *SpeI* into nine different PFGE patterns with predominance of the *SpeI01* and *SpeI02*. All NAR isolates had higher MICs for fluoroquinolone. We validate the use of the nalidixic acid screening test for detection of decreased fluoroquinolone susceptibility in SPA.

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

INTRODUCTION

Enteric fever caused by nalidixic acid-resistant (NAR) isolates have reduced susceptibility to fluoroquinolones, and in endemic countries, this is associated with higher rates of morbidity and mortality, particularly prolonged fever clearance time and increased need for retreatment of fever patients (Bhan et al., 2005; Molbak 2005; Le et al., 2004; Kadiravan et al., 2005; Crup et al., 2008). Although ciprofloxacin resistance (MIC \geq 4 μ g/ml), there have been frequent reports of reduced susceptibility to fluoroquinolones (ciprofloxacin MICs of 0.25 to 1.0 μ g/ml) among *Salmonella enterica* strains, is rare (Bhan et al., 2005; Molbak 2005; Le et al., 2004; Kadiravan et al., 2005; Crup et al., 2008; Dimitrov et al. 2007; Chandel et al., 2000; Hume et al. 2009).

The incidence of paratyphoid fever A caused by NAR strains at Yuxi city of China increased progressively after 1999, and the number of SPA isolates susceptible to ciprofloxacin markedly decreased. An increasing proportion of cases of enteric fever are due to SPA.

Isolates of SPA from Yuxi city, which cause 42% (8/19), 97% (73/75) and 99% (1262/1265) of culture-proven enteric fever cases in 1999, 2000 and 2001, have been increasing until December 2008 (Shukun et al., 2009; Fanlin et al., 2005; Shukun et al., 2008; Hongqian et al., 2004). We have studied a collection of nalidixic acid-resistant (NAR) and -susceptible (NAS) isolates of SPA to determine whether there is evidence to support the hypothesis that a high incidence of nalidixic acid resistance may arise from mutation/selection events with subsequent clonal expansion.

MATERIALS AND METHODS

Bacterial strains

A total of 4060 sporadic isolates of SPA were obtained from blood cultures of different individuals (peoples who lived in 7 separate areas in Yuxi) admitted with paratyphoid fever A to the hospitals and centers for disease control and prevention located in varied areas and epidemiologically independent to represent a wide geographic distribution within Yuxi between January 1999 and December 2008.

*Corresponding author E-mail: yxwsk@hotmail.com

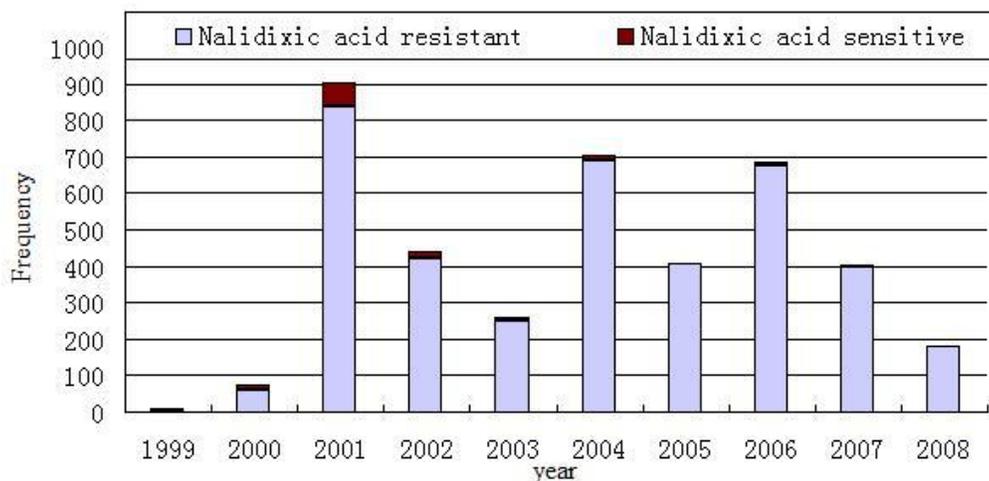


Figure 1. Frequency of nalidixic acid-resistant and –sensitive SPA isolates from Yuxi by year from 1999 to 2008

(give method reference with brief of the method used). All organisms isolated following subculture were identified to the serotype level by agglutination tests with *Salmonella* 'O': 2-and 'H': a specific antiserum (Biological products institute of Ministry of Public Health, Lanzhou, China) and standard biochemical tests (Automatic Microorganism Identification System VITEK 32 GNI+ or API 20E, bioMérieux, France) (Hendriksen 2003)

Antimicrobial susceptibility testing

All 4060 isolates were tested for antimicrobial susceptibility by the controlled Kirby-Bauer disc diffusion technique on Muller-Hinton agar (Oxoid, Basingstoke, United Kingdom) plates (CLSI 2009). The antibiotic disks (Oxoid Limited, Hampshire, England; the disk content is indicated in parentheses) contained nalidixic acid (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), amoxicillin (25 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftazidime (30 µg), imipenem (10 µg), sulphamethoxazole (25 µg), tetracycline (30 µg), and chloramphenicol (30 µg). One strain of *Escherichia coli* ATCC 25922 was used as control for potency of antibiotics.

A total of 186 isolates (166 NAR isolates and 20 NAS isolates) were randomly selected for further testing of their MICs of nalidixic acid, ciprofloxacin, levofloxacin, ofloxacin, norfloxacin, and gatifloxacin. The MICs were determined by the Etest (AB Biodisk, Solna, Sweden) (Hakanen et al., 2005). *Escherichia coli* ATCC 25922 was included as MIC controls.

PFGE

For subtyping of 166 NAR isolates and 20 NAS isolates, PFGE procedure available at PulseNet website of The Center for Disease Control and Prevention, the United States and published earlier (Gaul et al., 2007; Sandt et al., 2006; Hopkins et al., 2005) was followed using restriction endonucleases SpeI (TaKaRa Biotechnology Co., Ltd., Dalian, China). The plugs were run in a 1% (w/v) SeaKem Gold agarose gel using a CHEF-DR III Pulsed-Field System (Bio-Rad, the United States) in 0.5% Tris-borate-EDTA buffer (Sigma) at 10-14°C. 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 TIFF file format. The file images were processed by BioNumerics software version 4.6 (Applied

Maths, BVBA, Kortrijk, Belgium). XbaI-digested DNA from *Salmonella enterica* serovar Braenderup H9812 (ATCC BAA-664) was used as molecular size marker (Swaminathan et al., 2001).

RESULTS

Antimicrobial susceptibility testing

The 4060 isolates tested by the disc diffusion technique exhibited high incidence of resistance to nalidixic acid, 96.9% (3 933/4 060) of the isolates obtained from 1999 to 2008 were resistant to nalidixic acid, 61 resistant strains of 81 isolates between 1999 and 2000 were NAR. Out of 4 060 isolates during 2001 and 2008, 3 872 were NAR. NAS isolates predominated in 1999 but NAR after 2000, continuous surveillance has shown that the prevalence has been rising steadily and that, at present, more than 99.5% of all SPA isolates are NAR. The increase was especially striking among isolates obtained from 2001 (Figure 1). Comparison of the total number of NAR strains among the ten collection years revealed a dramatic increase in the percentage, from 12.5% (1/8) in 1999 to 100% (182/182) in 2008 ($P < 0.001$). All the isolates tested were susceptible to cefotaxime, ceftazidime, tetracycline, and chloramphenicol. Less than 0.4% (16/4060) of isolates obtained from 1999 to 2008 were resistant to imipenem, less than 2.0% (81/4060) of isolates were resistant to sulphamethoxazole, and 15.0% (609/4060) of isolates were resistant to ampicillin and amoxicillin.

The phenotype of NAR or NAS isolates determined by disk diffusion was further confirmed by the Etest, clearly indicating that the resistance to nalidixic acid might be used as a marker for reduced fluoroquinolones susceptibility. Although most NAR isolates were susceptible to many antibiotics of fluoroquinolones

Table 1. MICs($\mu\text{g/ml}$) of nalidixic acid-resistant and -susceptible SPA isolates^a

Resistance to nalidixic acid	Strains studied	MIC ($\mu\text{g/ml}$)				
		Ciprofloxacin	Levofloxacin	ofloxacin	Norfloxacin	Gatifloxacin
Resistant isolates	166	0.5 (77)	0.5 (22)	1.5 (37)	2 (8)	0.38 (11)
		0.7 (89)	0.75 (88)	2.0 (96)	3 (103)	0.50 (118)
		MIC > 256 $\mu\text{g/ml}$	1.0 (52)	3.0 (15)	4 (55)	0.75 (37)
Susceptible isolates	20	0.032 (12)	0.094 (12)	0.25 (5)	0.125 (4)	0.047 (12)
		0.030 (4)	0.032 (8)	0.19 (12)	0.025 (16)	0.023 (8)
		MIC \leq 16 $\mu\text{g/ml}$	0.023 (4)		0.125 (3)	

^aNumbers in table cells represent MICs; Numbers in parentheses refer to the number of isolates

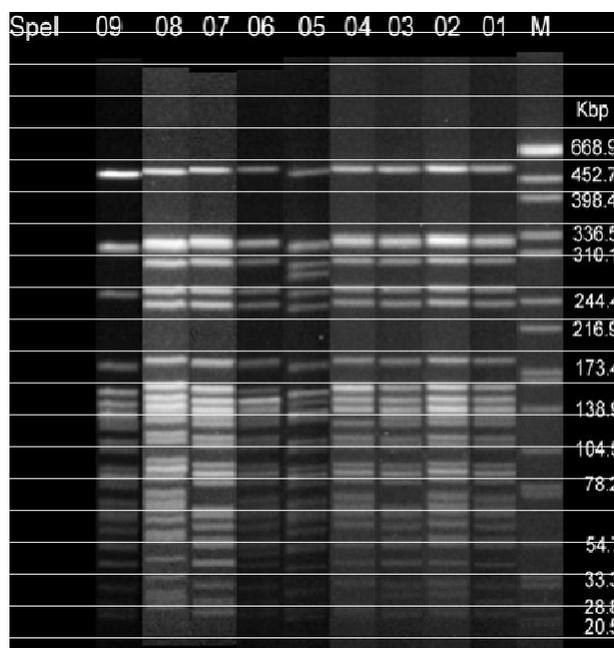


Figure 2. PFGE patterns from representative strains of SPA collected from patients following digestion with SpeI. Pattern SpeI01 and SpeI02: epidemic clone in Yuxi region.

according to the CLSI criteria, the MICs were higher than that for the sensitive isolates, and a significant correlation ($P < 0.001$) between nalidixic acid resistance and an elevated fluoroquinolones MICs was observed (Table 1).

PFGE

Analysis by PFGE of restriction fragments from the SpeI digestion of genomic DNA from 186 isolates of SPA produced 9 distinct patterns (Figures 2, and 3, table 2). The application of PFGE to SPA isolates from peoples who lived in 7 separate areas in Yuxi showed that the predominant isolates were virtually identical, and this

strain type seems to be predominant in the 7 study areas examined here. PFGE pattern SpeI01 and SpeI02 consisted of 45.2% (84/186) and 40.3% (75/186) of isolates, respectively. Pattern SpeI03 consisted of 2.2% (4/186) of isolates, SpeI04 consisted of 0.54% (1/186) of isolates. Pattern SpeI05, SpeI07 and SpeI09 consisted of 1.1% of isolates, respectively. Pattern SpeI08 consisted of 3.2% (6/186) of isolates. Nine patterns which differed in only 1 to 3 bands were shared among the isolates indicating that these isolates were closely related. The 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

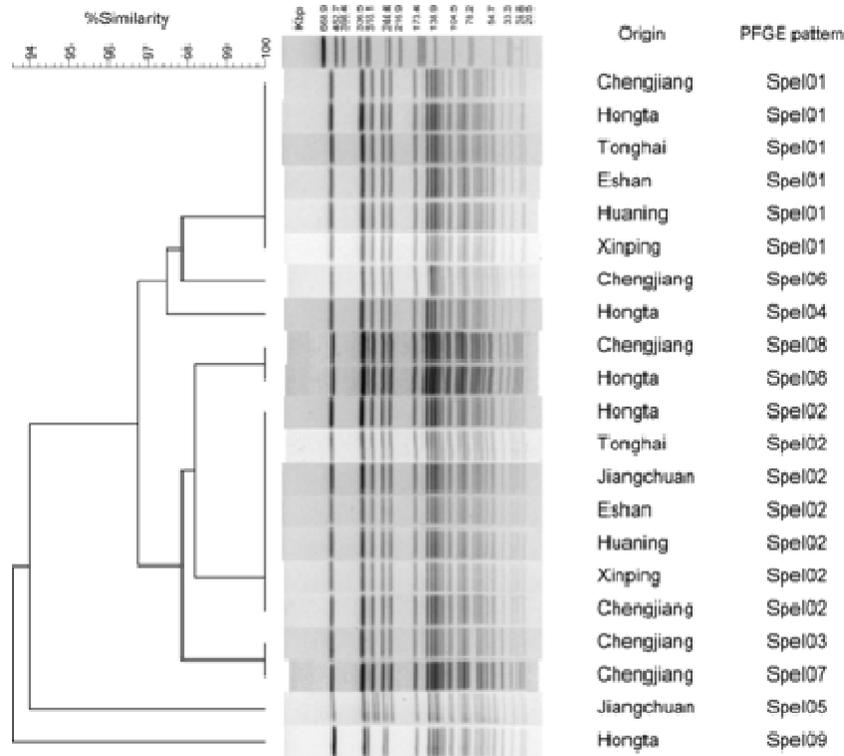


Figure 3. Cluster analysis of the PFGE SpeI fragment pattern of SPA from representative strains of SPA collected from patients.

(profiles differed by one or more bands of >100 kb) was observed among group SpeI05 and SpeI09 isolates. Among the various PFGE patterns identified, most isolates belonged to 1-2 predominant patterns, pattern SpeI01 and SpeI02, the main cause of the epidemics, is highly prevalent in humans in 7 counties of Yuxi. Surveillance of 186 SPA isolates demonstrated a surge in PFGE pattern SpeI01 and SpeI02 isolates. The other 7 PFGE patterns isolates tested did not match the epidemic patterns.

Nine clusters of the 186 SPA isolates were identified by PFGE (Figures 2, 3, and table 2). Based on the 93.6% similarity, there were 2 clusters. The 2 isolates from Jiangchuan were grouped into one cluster which shared pattern SpeI05, the 1 isolate from Hongta shared pattern SpeI09. Based on the 98.0% similarity, there were 2 major clusters. The 84 isolates from Chengjiang, Hongta, Tonghai, Eshan, Huaning, and Xinping were grouped into one major cluster which shared pattern SpeI01, the 15 isolates from Chengjiang were shared pattern SpeI06. Based on the 98.2% similarity, there were 2 major clusters. The 75 isolates from Hongta, Tonghai, Jiangchuan, Eshan, Huaning, Xinping and Chengjiang were grouped into one major cluster which shared pattern SpeI02, the 3 isolates from Hongta and Chengjiang were grouped into another cluster which shared pattern SpeI08. In contrast, the 12 sporadic isolates from Chengjiang, Hongta, and Jiangchuan gave PFGE patterns (SpeI03, SpeI04, SpeI05,

SpeI06, SpeI07, SpeI08, SpeI09) which were unique and distinctly different from one another. Of 186 isolates, it is interesting to compare the earlier isolates with the latter isolates, in particular to compare with NAR isolates, to see that there had been some changes in predominant PFGE patterns over the time. SpeI02 isolates predominated between 1999-2000 but were gradually replaced by SpeI01 isolates after 2000 (Table 2).

Antibiotic susceptibility and PFGE pattern

Results of antibiotic susceptibility testing and PFGE support the existence of distinct groups of SPA in population. Resistance to nalidixic acid was not associated with a particular PFGE pattern and PFGE was able to subtype 166 NAR strains into seven groups (SpeI01, SpeI02, SpeI03, SpeI04, SpeI07, SpeI08, SpeI09). There were three PFGE patterns (SpeI01, SpeI05, SpeI06) among 20 NAS strains. PFGE was a useful subtyping technique to differentiate SPA. However, it fails to differentiate between antimicrobial-resistant and -sensitive strains.

DISCUSSION

In the study SPA was highly prevalent and remains

Table 2. Changes in PFGE patterns of the earlier and latter SPA isolates from Yuxi

Years of origin	Resistance to NA*	Strains studied	PFGE pattern (Spel)								
			01	02	03	04	05	06	07	08	09
1999-2000	NAR	35	9	24	1	1	0	0			
	NAS	5	2	0	0	0	1	2			
2001-2002	NAR	25	12	13			0	0			
	NAS	5	1	0			1	3			
2003-2004	NAR	35	18	16	1			0			
	NAS	3	0	0	0			3			
2005-2006	NAR	28	16	12				0			
	NAS	5	0	0				5			
2007-2008	NAR	43	26	10	2			0	1	3	1
	NAS	2	0	0	0			2	0	0	0
Total		186	84	75	4	1	2	15	1	3	1

*NA: nalidixic acid;
 NAR: nalidixic acid resistant;
 NAS: nalidixic acid susceptible.

endemic in Yuxi. NAR SPA epidemic occurred in Hongta after 1999 and NAR strains have been isolated from all Yuxi counties (Ref?). But such a high prevalence of resistance had not been reported in earlier studies (Shukun et al., 2009; Fanlin et al., 2005; Shukun et al., 2008; Hongqian et al., 2004; Solnik-Isaac et al., 2007; Murray et al; 2003). We screened for mutations in *gyrA*, *gyrB*, *parC* and *parE* genes within the quinolone resistance-determining region (QRDR) to target the reason for the high incidence of nalidixic acid 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 (Shukun et al., 2008). To the best of our knowledge, a stable frequency of more than 96.9% (3 933/4 060) nalidixic acid resistance with MIC > 256 µg/ml and with elevated 5 fluoroquinolones MICs in SPA isolates over a 10-year study period had not been described with *Salmonella* (Hume et al., 2009;Threlfall et al., 2006). This rate of quinolone resistance was by far

higher than the rates reported for other *Salmonella* strains (Solnik-Isaac et al., 2007; Murray et al; 2003). The study validated the use of the nalidixic acid screening test in the detection of decreased fluoroquinolone susceptibility in SPA. Among 186 SPA isolates included in this study, which obtained from varied areas and epidemiologically independent to represent a wide geographic distribution within Yuxi, identification of nalidixic acid resistance by the disk diffusion method provided a sensitivity and specificity of 100% to screen for isolates with reduced susceptibility. The most isolates were found to be sensitive to all antibiotics tested except quinolones and fluoroquinolones. This can be explained by the fact that resistance to other antibiotics is plamid-mediated, and is independent of resistance to fluoroquinolones, which is chromosomal-based (Giraud et al., 2006).

Among the various PFGE patterns identified, most isolates belonged to 2 predominant patterns, pattern Spel01 and Spel02, the main cause of the epidemics, is highly prevalent in humans in 7

counties of Yuxi. 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 fluoroquinolones. Spel03, Spel04, Spel05, Spel06, Spel07, Spel08, and Spel09 isolates tested did not match the epidemic patterns/clones and were considered to be unrelated sporadic patterns or occurrence of microevolution (generation-to-generation small-scale genetic changes in a population) within the epidemic patterns/clones (Le et al., 2007). On the basis of PFGE results it can be assumed that most of the paratyphoid fever A cases which occurred over ten years in Yuxi were due to the clonal expansion of two predominant clones coexisted in Yuxi, since 1999 and even before. If the distinction is not reliable (why?), and taking into account a proportional part of the other strains or the occurrence of microevolution within the clone (Le et al., 2007). 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 is also supported by the observations that most isolates had closely related PFGE profiles. Our data suggest that the high prevalence of NAR with increased ciprofloxacin, levofloxacin, ofloxacin, norfloxacin, and gatifloxacin MICs among SPA isolates in Yuxi probably emerged from the spread of a resistant clone with the Ser83Phe switch that had a single mutation in the *gyrA* gene (Shukun et al., 2008). The strains profited by clonal expansion under pressure of the classical first-line antibiotics, and maintained a chromosomal point mutation under fluoroquinolone pressure (how are you sure that it existed, is there any reference of indiscriminate or excessive use of fluoroquinolones?) and extended clonal expansion. 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 2008.

The present study expands the evidence supporting clonal expansion as a major contributor to a high incidence of nalidixic acid resistance in SPA. One would predict that clonal expansion would result in the NAR organisms being relatively homogeneous, whereas the frequent mutation/selection hypothesis should result in greater heterogeneity (Bertrand et al., 2006; Kilmartin et al., 2005). Continued monitoring of antimicrobial resistance and clonal expansion 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 nalidixic acid resistance (Stock and Wiedemann, 2000). Suitable measures in the field of antibiotics administration are needed to prevent increasing levels of resistance to fluoroquinolone, in particular regarding the reuse of classical antibiotics such as chloramphenicol, cotrimoxazole, azithromycin and cephalosporins. As fluoroquinolone resistance is chromosomally mediated, the chances of it spreading horizontally are reduced. However, selective pressures exerted by the overuse of these drugs may see such isolates becoming more common in the future. The high rate of NAR isolates from Yuxi presents a strong case for initiating non-fluoroquinolone-based therapy for patients with suspected enteric fever. Recommendation for the emergency treatment of outbreaks and epidemics caused by a fully fluoroquinolone-resistant strain can be made. The health authority should target the fully fluoroquinolone-resistant clones for the application of more intensive and efficacious control measures. 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.

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