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Pulsed-Field Gel Electrophoresis and Mutation Typing of *gyrA* Gene of Quinolone-Resistant *Salmonella enterica* Serovar Paratyphi A Isolated from Outbreak and Sporadic Cases, 1998–2002, Korea

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Abstract In early 2002, over 200 people in the city of Pusan. Korea suffered from paratyphoid fever resulting from *Salmonella* Paratyphi A infection. Antimicrobial susceptibility tests and *Xba*I pulsed-field gel electrophoresis (PFGE) were conducted to 54 *Salmonella* Paratyphi A isolated from humans during the period of 1998 to 2002. Most of the isolates (83%) were only nalidixic acid-resistant and 78% were X 1 PFGE patterns. Also, we measured the MIC of ciprofloxacin and screened *gyrA* mutation(s) using allele-specific PCR and restriction fragment length polymorphism (AS-PCR-RFLP). The representative 5 isolates in 2002 and 1 isolate in 2000 were 1 μg/ml of MIC and had mutation at the 83rd codon in *gyrA*. These data suggest that the outbreak in the early 2002 might have been due to dissemination of the strain present in 2000. Also, decreased susceptibility to ciprofloxacin was partly due to the mutation at the 83rd codon in *gyrA*.

Key words: *Salmonella* Paratyphi A, pulsed-field gel electrophoresis, resistance, quinolone, nalidixic acid, *gyrA*, mutation

Salmonella Paratyphi A is one of the *Salmonella* serovars responsible for paratyphoid fever, which is a serious, contagious disease in humans. Of the 1,000–2,000 salmonellosis cases per year reported in Korea during 1994–2001, about 0.5% of the cases were paratyphoid fever [our unpublished data]. Paratyphoid fever is caused by three strains of *Salmonella* Paratyphi: *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, and *Salmonella* Paratyphi C. It can be transmitted through animals, contaminated foods and water, and humans to humans. The incubation period is one to two weeks, but could be different depending on age [10, 13]. The reported outbreaks by *Salmonella* Paratyphi A infection are rare worldwide. In India and Singapore, outbreaks of food-associated *Salmonella* Paratyphi A have been reported [3, 14, 24, 25].

Quinolone antibiotics constitute a treatment of choice in instances of acute salmonellosis, therefore, the emergence of quinolone-resistant pathogenic *Salmonella* strains is a serious health problem [2, 5, 23], and failure of cases to treat because of quinolone resistant *Salmonella* Typhi, have been reported [26, 27]. Chandel *et al.* [4] reported that *Salmonella* Paratyphi A has been increasing in India since 1996, and 32% of isolates from the New Delhi region had decreased susceptibility to ciprofloxacin (MIC >2.0 mg/l). Hirose *et al.* [12] reported that 5.4% of *Salmonella* Paratyphi A isolates exhibited reduced susceptibility to ciprofloxacin and had a point mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene.

In early 2002, an outbreak resulting from *Salmonella* Paratyphi A infection occurred in Pusan, the 2nd biggest metropolitan city in Korea. The number of patients was over 200. Furthermore, other paratyphoid patients who suffered from high fever, headache, chill, and diarrhea were also reported nationwide after the Pusan outbreak. Epidemiological investigation proved that the outbreak in Pusan was caused by water-borne paratyphoid fever, resulting from a contaminated water-supply system. Most patients were cured by treatment with ciprofloxacin, and ceftriaxone was also used when some patients treated with ciprofloxacin still suffered from continued high fever.

A total of 54 isolates of *Salmonella* Paratyphi A were tested in the present study. Forty isolates out of all tested *Salmonella* Paratyphi A were collected from outbreaks in Pusan (30 isolates) or other areas (10 isolates) during 2002 in Korea. The remaining 14 isolates were collected from sporadic cases during 1998–2001 in Korea. In order

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Table 1. Antibiograms and PFGE patterns of *Salmonella* Paratyphi A isolates, 1998–2002.

Year	No.	Antibiogram[a] (No.)	PFGE pattern (No.)	Remark
1998	3	NA (1), Susceptible (2)	X 2 (3)	Sporadic cases
1999	1	NA (1)	X 3 (1)	Sporadic case
2000	3	NA (2), Susceptible (1)	X 1 (2), X 2 (1)	Sporadic cases
2001	7	NA (1), K (1), Susceptible (5)	X 2 (7)	Sporadic cases
2002	30	NA (30)	X 1 (30)	Outbreak in Pusan city
2002	10	NA (10)	X 1 (10)	Outbreak outside Pusan city

[a]NA=nalidixic acid, K=kanamycin, Susceptible=susceptible to all antibiotics tested.

to characterize these *Salmonella* Paratyphi A isolates, antimicrobial susceptibility tests and *Xba*I pulsed-field gel electrophoresis (PFGE) were performed. We also conducted the minimum inhibitory concentration (MIC) test of the isolates to ciprofloxacin. Furthermore, to study resistance characteristics against quinolone antibiotics, the gyrase mutation assay with the representative 10 isolates was conducted using allele-specific PCR and restriction fragment length polymorphism (AS-PCR-RFLP).

All isolates were identified and confirmed by biochemical and serological tests, using API 20E (bioMerieux, France) kit and sera from Difco (Detroit Michigan, U.S.A.), respectively.

The strains were tested for their antibiotic susceptibility on Mueller-Hinton agar plates by the disk diffusion method [18]. The media and disks were purchased from BBL (Becton Dickinson Microbiology Systems, Cockeysville, U.S.A.). Resistance to the following antibiotics was tested with disks containing: ampicillin 10 μg, chloramphenicol 30 μg, gentamicin 10 μg, streptomycin 10 μg, tetracycline 30 μg, nalidixic acid 30 μg, ciprofloxacin 5 μg, ceftriaxone 30 μg, cefotaxime 30 μg, kanamycin 30 μg, sulfamethoxazole/trimethoprim 23.75 μg/1.25 μg, ampicillin/sulbactam 20 μg, ticarcillin 75 μg, cefoxitin 30 μg, amoxacillin/clavulanic acid 30 μg, and amikacin 30 μg. Susceptibility of the isolates to antibiotics was interpreted by measuring the inhibition zones, as recommended by the supplier, except that the intermediate and sensitive isolates were grouped together. *Escherichia coli* ATCC 25922 was used as a reference strain for quality control [15].

MIC determinations were performed on Mueller-Hinton agar (Difco) according to the standards of the National Committee for Clinical Laboratory Standards [17, 22]. Ciprofloxacin HCl (U.S. Pharmacopeia, Rockville, U.S.A.) was used as a fluoroquinolone antibiotic.

We assayed *gyrA* mutations at codons 81, 83, and 87, using allele-specific PCR and restriction fragment length polymorphism (AS-PCR-RFLP), described by Giraud *et al.* [7]. This assay was performed to screen the *gyrA* mutations of the representative 10 nalidixic acid-resistant *Salmonella* Paratyphi A strains isolated during the period from 1998 to 2002.

The preparation of genomic DNA blocks and digestion with restriction enzyme were carried out as described by Gautom *et al.* [6, 19]. All *Salmonella* Paratyphi A isolates were analyzed by using restriction enzymes *Xba*I (New England Biolabs, Beverly, MA, U.S.A.). Typing by PFGE of genomic DNA digested with *Xba*I was carried out in a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The PFGE pulsing and running conditions were an initial 2.2 seconds to a final 63.8 seconds for 18 h and 6 Volts/cm at 14°C. Lambda ladder (New England Biolabs) was used as a molecular size marker. After electrophoresis, the gels were stained with ethidium bromide for 20 min and were photographed using Gel Doc 2000 (Bio-Rad Laboratories).

Fifty-four *Salmonella* Paratyphi A isolates were tested for identification of antimicrobial susceptibility pattern. All isolates in 2002 were resistant to nalidixic acid only, as were 5 out of 14 isolates during 1998–2001. One isolate in 2001 was resistant to kanamycin only. The remaining 8 isolates were susceptible to all 16 antibiotics tested (Table 1).

In order to determine the MIC of ciprofloxacin on nalidixic acid-resistant isolates, we selected and tested the representative 10 isolates, which included 5 isolates in 2002 and 5 isolates during 1998–2001. Interestingly, all 5 isolates in 2002 and 1 isolate in 2000 had high level of resistance (1 μg/ml MIC), and the remaining 4 isolates had very low level of resistance (≤0.06 μg/ml MIC). Furthermore, the results of mutation analysis by AS-PCR-RFLP showed the same pattern as MIC data; all 5 isolates in 2002 and 1 isolate in 2000 were mutated at the 83rd codon of *gyrA* and the remaining 4 isolates were mutated at the 87th codon of *gyrA* (Table 2).

For the purpose of comparing genetic clonality and chasing the original outbreak strain, we performed PFGE with the restriction enzyme, *Xba*I. As listed in Table 1, the PFGE patterns of all 40 isolates in 2002 and 2 isolates in 2000 were X 1, while the remaining isolates were X 2 patterns, except one isolate in 1999 (X 3). Figure 1 shows each PFGE pattern.

Enteric fever caused by *Salmonella* Paratyphi A infection was less frequent in Korea and worldwide than by other *Salmonella* serovars. There was no big outbreak resulting from *Salmonella* Paratyphi A infection in Korea during our surveillance of enteric bacteria. There were reported

Table 2. PFGE patterns, MICs of ciprofloxacin, and mutation assay results of *gyrA* gene for representative 10 nalidixic acid-resistant strains.

Strains (yr)	PFGE pattern	CIP[a] MIC (μg/ml)	Codon No. of mutation in *gyrA* detected by AS-PCR-RFLP
KJ567 ('02)	X 1	1	83
KJ568 ('02)	X 1	1	83
KJ569 ('02)	X 1	1	83
KJ570 ('02)	X 1	1	83
KJ571 ('02)	X 1	1	83
KJ2144 ('01)	X 2	≤0.06	87
KJ301 ('00)	X 1	≤0.06	87
KJ394 ('00)	X 1	1	83
KJ476 ('99)	X 3	≤0.06	87
KJ104 ('98)	X 2	≤0.06	87

[a]CIP=ciprofloxacin.

outbreaks by *Salmonella* Paratyphi A infection in India and Singapore [3, 14, 24, 25].

PFGE is a useful subtyping method to differentiate *Salmonella* Paratyphi A [8]; therefore, we performed *Xba*I PFGE as a molecular epidemiological marker. *Salmonella* Paratyphi A isolates obtained from the Pusan city outbreak and isolates from other areas in 2002 had the same *Xba*I PFGE pattern (X 1) as those of 2 isolates in 2000. One of these two isolates in 2000 had the same MIC value for ciprofloxacin and *gyrA* mutation pattern as the isolates in 2002. These data strongly suggest that the emergence of *Salmonella* Paratyphi A in 2002 probably was derived from the dissemination of X 1 pattern and *gyrA* 83rd codon-mutated isolate (KJ394) in 2000 (Table 2). Most of the patients in 2002 who lived in the non-Pusan area had visited Pusan city in early 2002. Epidemiological investigators proved that the outbreak in Pusan city was caused by a contaminated water-supply system. We are not certain how the X 1 pattern *Salmonella* Paratyphi A strain found in 2000 emerged in the Pusan water-supply system.

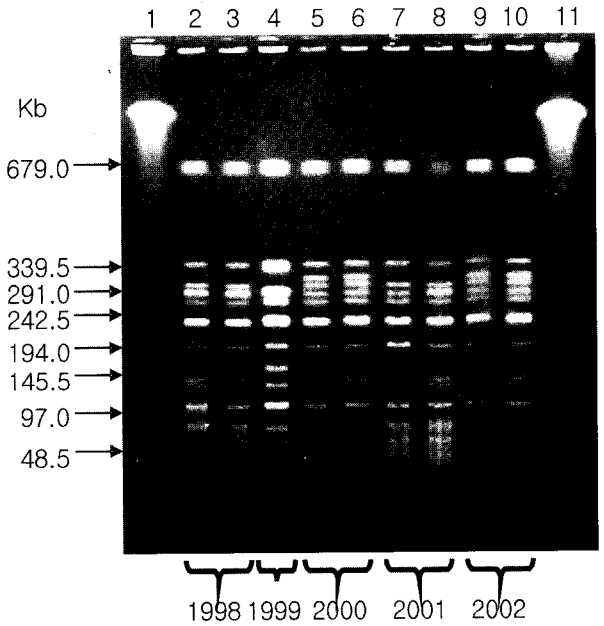

Fig. 1. Representative *Xba*I PFGE patterns of *Salmonella* Paratyphi A isolated from Korean patients.
Lanes 2, 3 show the pattern of isolates obtained in 1998. Lane 4 shows the pattern of an isolate obtained in 1999. Lanes 5, 6 show the pattern of isolates obtained in 2000. Lanes 7, 8 show the pattern of isolates obtained in 2001. Lanes 9, 10 show the pattern of isolates obtained in 2002. Lanes 1 and 11 are lambda phage DNA size markers. X 1 patterns are lanes 5, 6, 9, 10. X 2 patterns are lanes 7, 8 and X 3 pattern is lane 4.

Most *Salmonella* Paratyphi A isolates (83%) were resistant to nalidixic acid. In cases of *Salmonella* Typhi and other serovars, nalidixic acid- and ciprofloxacin-resistant strains increased dramatically [1, 11, 16, 20, 26]. In India, nalidixic acid-resistant *Salmonella* Typhi with decreased susceptibility to ciprofloxacin was endemic and treatment failures increased [26]. Like cases in India, similar phenomena were reported in the treatment of *Salmonella* Paratyphi A infection with ciprofloxacin. When ceftriaxone was used, the patients were apyrexial. *Salmonella* serovars with decreased susceptibility had a point mutation in the quinolone resistance-determining region of the *gyrA* gene [7, 9, 21]. According to our AS-PCR-RFLP results, decreased susceptibility of ciprofloxacin was at least due to the mutation at the 83rd codon in *gyrA*, and the isolates with mutation at the 87th codon were not resistant to ciprofloxacin but to nalidixic acid.

Emerging nalidixic acid-resistant *Salmonella* Paratyphi A is a serious challenge to Korean public health. We speculate that increasing salmonellosis, including paratyphoid in Korea, has partly resulted from elevated average yearly temperature. With nations with emerging paratyphoid, surveillance cooperation is greatly needed. New standardization for the use and treatment of antibiotics is also needed in all countries including Korea.

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