Jpn. J. Infect. Dis., 65, 264-267, 2012

Short Communication

Antibiotic Susceptibility Pattern and the Indicator of Decreased Ciprofloxacin Susceptibility of *Salmonella enterica* Serovar Typhi Isolated from Dhulikhel Hospital, Nepal

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(Received December 28, 2011. Accepted April 4, 2012)

SUMMARY: Monitoring the antibiotic susceptibility pattern of *Salmonella enterica* serovar Typhi (*S.* Typhi) is important for efficiently managing cases of typhoid fever. In this study, the antimicrobial susceptibility patterns of 114 *S.* Typhi isolates, which were collected from a university hospital in Nepal during July 2009–December 2010, were investigated by disc diffusion assays. All of the *S.* Typhi isolates were sensitive to amoxycillin-clavulanic acid. More than 95% of the isolates were sensitive to chloramphenicol, ceftazidime, ceftriaxone, and cotrimoxazole. In addition, 1.7% of the studied isolates showed multiple drug resistance patterns. Of the 40 *S.* Typhi isolates, 32 strains (80%) showed nalidixic acid (NA) resistance with decreased susceptibility to ciprofloxacin (CIP). Importantly, we found the simultaneous presence of NA resistance and decreased susceptibility to CIP, suggesting that the resistance to NA is a reliable indicator of decreased CIP susceptibility (sensitivity, 97.5%; specificity, 100.0%). Furthermore, the sequencing of NA-resistant *S.* Typhi isolates showed a predominant amino acid alteration in the quinolone resistance-determining region (QRDR) of *gyrA* gene at position 83 from Ser→Phe. Two isolates with resistance to both CIP and NA had a double-mutation (Ser83→Phe and Asp87→Asn) in the QRDR of the *gyrA* gene, of which one had an additional amino acid mutation (Ser80→Ilu) in the QRDR of the *parC* gene.

Typhoid fever, which is a systemic infection caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), is a major health problem in developing countries, including Nepal (1). There are approximately 21.6 million cases of typhoid fever worldwide and an estimated 200,000 deaths every year (2,3). The disease, which is transmitted through the fecal-oral route, can be treated with antibiotics. Problems with effective treatment arose in the 1980s with the discovery of multidrug-resistant (MDR) *S.* Typhi, which were resistant to all 3 antityphoidal antimicrobial agents, namely ampicillin, chloramphenicol and cotrimoxazole (4). Because of the rise in MDR strains, fluoroquinolones have become the treatment of choice for typhoid fever. Unfortunately, *S.* Typhi strains with reduced susceptibility to fluoroquinolones have also been reported in several countries, such as Vietnam (2), Nepal (4), India (5), and Bangladesh (6). Therefore, the genetic basis of fluoroquinolone resistance in *S.* Typhi has been widely investigated. A point mutation in the quinolone resistance-determining region (QRDR) of the bacterial DNA gyrase and/or DNA topoisomerase IV is the most common mechanism leading to decreased susceptibility to fluoroquinolones (9,10). In Nepal, there have been several studies that have focused on the prevalence of MDR and the antibiotic susceptibility patterns of *S.* Typhi (4,7,8). Importantly, the antibiotic susceptibility pattern of bacteria can fluctuate spatially and temporally. There is a need to monitor the antibiotic-resistance patterns of *S.* Typhi in order to help guide treatment policies in affected countries. In addition, we investigated the relevance of using nalidixic acid (NA) resistance as a marker of fluoroquinolone susceptibility and the molecular mechanisms leading to a decreased susceptibility to fluoroquinolones in *S.* Typhi.

A total of 114 *S.* Typhi isolates were isolated from blood samples of outpatients and inpatients visiting Dhulikhel Hospital-Kathmandu University Hospital (DH-KUH), which is a regional teaching hospital (350 beds) that is located in central Nepal and that is focused on serving rural communities. Bacterial identifications were done using standard biochemical tests, and serotyping was performed by slide agglutination tests using specific *Salmonella* anti-Vi and anti-D antisera (Clinag, Bangkok, Thailand). Antibiotic susceptibilities were determined using the Kirby-Bauer disc diffusion method. The antibiotic discs (Oxoid, Basingstoke, England) included ampicillin (10 μg), ciprofloxacin (CIP) (5 μg), ofloxacin (5 μg), NA (30 μg), cotrimoxazole (25 μg), chloramphenicol (30 μg), amoxycillin-clavulanic acid (30 μg), cefotaxime (30 μg), ceftazidime (30 μg),

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and ceftriaxone (30 μg). *Escherichia coli* ATCC 25922 was included as a control.

The results of these antibiotic susceptibility tests are shown in Table 1. Of the 114 *S.* Typhi isolates, 100% were sensitive to amoxycillin-clavulanic acid and more than 95.5% of the isolates were sensitive to chloramphenicol, cotrimoxazole, ceftriaxone, or ceftazidime. In contrast, only 22.8% (26 strains) of the isolates were sensitive to NA, indicating a high occurrence of NA resistance in *S.* Typhi. Indeed, the highest resistance rate (77.2%) among the antibiotics tested was for NA.

Towards the end of the 1980s and 1990s, MDR *S.* Typhi strains were identified, and outbreaks of infections with these strains occurred in India, Pakistan, Bangladesh, Vietnam, the Middle East, and Africa (1). In the present study, only 2 isolates (1.8%) were found to be resistant to ampicillin, chloramphenicol, and cotrimoxazole. Despite the low number of total isolates included in this study, these observations seem to reflect the decreasing trend in identified MDR *S.* Typhi, which correlates with previous studies. For example, Khanal et al. (4) reported finding 26.5% MDR isolates during the study period of 2000 to 2004 in Eastern Nepal. However, by 2004, this percentage had begun to drop (4). The same trend has been noted in India, Bangladesh, and Central Nepal (5,7,8,11). The current low frequency of MDR *S.* Typhi isolates that have been discovered suggests that it may be possible to use chloramphenicol (98.2% sensitive) and cotrimoxazole (97.4% sensitive) again for the treatment of enteric fever.

Both CIP and NA belong to the quinolone antibiotic group. The identification of significant numbers of *S.* Typhi with complete resistance to NA and intermediate resistance to CIP (Table 1) led us to quantitatively determine the antibiotic susceptibility to these quinolones. Forty isolates were randomly selected that included mostly those with NA and CIP resistance, some NA-susceptible isolates, and some with intermediate-susceptible isolates to CIP. They were assayed by the Muller-Hinton agar dilution method in order to identify the minimum inhibitory concentrations (MIC) of CIP and NA and interpreted according to CLSI 2012 guidelines. The MICs for those susceptible and resistant to NA were ≤ 16 mg/L and ≥ 32 mg/L, respectively. The MIC for those susceptible, intermediate, and resistant to CIP were ≤ 1 mg/L, 2 mg/L and ≥ 4 mg/L, respectively, whereas the decreased CIP susceptibility was 0.125–1.0 mg/L.

Table 1. Antimicrobial susceptibility pattern of 114 *S.* Typhi isolates detected by Kirby-Bauer disc diffusion assay

Antimicrobial agent	Sensitive	Intermediate	Resistant
	% (no.)	% (no.)	% (no.)
Amoxycillin-clavulanic acid	100.0 (114)	—	—
Chloramphenicol	98.2 (112)	—	1.8 (2)
Cotrimoxazole	97.4 (111)	—	2.6 (3)
Ceftriaxone	96.5 (110)	3.5 (4)	—
Ceftazidime	95.6 (109)	4.4 (5)	—
Ofloxacin	91.2 (104)	7.0 (8)	1.8 (2)
Cefotaxime	87.8 (100)	9.6 (11)	2.6 (3)
Ciprofloxacin	79.8 (91)	18.4 (21)	1.8 (2)
Ampicillin	67.5 (77)	2.6 (3)	29.8 (34)
Nalidixic acid	22.8 (26)	—	77.2 (88)

Among the 40 *S.* Typhi isolates, 34 isolates (85%) were NA resistant (MIC ≥ 32 mg/L), whilst the remaining 6 isolates (15%) were NA susceptible (Table 2). Notably, among the 34 NA-resistant isolates, almost all of them (32 isolates) had decreased CIP susceptibilities (ranging from 0.125 to 0.5 mg/L; Table 2). None of them was CIP susceptible. This result indicates a correlation between *S.* Typhi isolates with decreased CIP susceptibility and NA resistance. The calculated sensitivity and specificity of the correlation were 97.5% and 100%, respectively. Quantitatively determining the antibiotic MICs of the clinical isolates is not practical in routine clinical laboratories because it is a time consuming process, and requires experienced personnel. Most laboratories use the disc diffusion assay, which is a qualitative assay and which is not sufficiently sensitive to screen *S.* Typhi with decreased CIP susceptibility. Findings from this study and others (4,17) on the correlation between the resistance to NA and the decreased susceptibility to CIP will have important applications in clinical laboratories. The NA disc diffusion assay should be used as an indicator for detecting *S.* Typhi isolates with decreased CIP susceptibility.

The findings from this study of *S.* Typhi isolates with decreased susceptibility or resistance to CIP suggest that there is a potential for an increasing number of resistant strains against this group of drugs in Nepal. Noticeably, we have identified a number of isolates that are resistant to cefotaxime (third-generation cephalosporin). Researchers in Bangladesh, Pakistan, the Philippines, and India have reported the occurrence of *S.* Typhi that are resistant to ceftriaxone (12–15). If this trend of emerging cephalosporin resistance is particular to developing

Table 2. Correlation between the CIP susceptibility (detected by MIC) and NA resistance (detected by disc diffusion) among *S.* Typhi isolates

NA susceptibility[1)]	MICs of CIP (mg/L)									
	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8
NASST (6)	2	0	3	0	1	0	0	0	0	0
NARST (34)	0	0	0	24	6	2	0	0	1	1
Total (40)	2	0	3	24	7	2	0	0	1	1

[1)]: Detected by disc diffusion assay.

The interpretation for MIC of CIP and NA susceptibility were based on CLSI 2012 guidelines.

CIP, ciprofloxacin; NA, nalidixic acid; NASST, nalidixic acid-susceptible *S.* Typhi; NARST, nalidixic acid-resistant *S.* Typhi.

nations, antimicrobial resistance must be monitored, constantly keeping in mind that, after fluoroquinolones, third-generation cephalosporins are the only drug of choice for the treatment of typhoid fever.

In order to investigate the mechanisms of fluoroquinolone resistance, different characteristics of NA and CIP *S.* Typhi-susceptible isolates were selected for sequencing. There were 9 NA-resistant *S.* Typhi isolates with decreased CIP susceptibility, 2 isolates (ST45 and ST104) that were resistant to both NA and CIP, and 2 NA-sensitive *S.* Typhi isolates (ST77 and ST79 as control) (Table 2) that were subjected to the sequencing of the QRDR of the *gyrA* and *parC* genes. The QRDR of these 2 genes were selected for sequencing because a single mutation of the *gyrA* gene was reported to be associated with decreased CIP susceptibility. However, a high level of resistance is built up by a mutation in the QRDR of *parC* (10,18). Amplifications of *gyrA* (347 bp) and *parC* (270 bp) DNA in the QRDR were performed according to Chau et al. (2). The forward and reverse primer sequences were 5′-TGTCCGAGAT GGCCTGAAGC-3′ (GYRA/P1) and 5′-TACCGT CATAGTTATCCACG-3′ (GYRA/P2), respectively, for *gyrA* and 5′-CTATGCGATGTCAGAGCTGG-3′ (Stmparc1) and 5′-TAACAGCAGCTCGGCGTATT-3′ (Stmparc2), respectively, for *parC*. DNA sequencing was conducted by the dideoxynucleotide chain termination method using an automated DNA sequencer. The nucleotide sequences were analyzed by BLAST at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih. gov/Blast.cgi).

Table 3 shows that there were 4 different types of mutation in the QRDR of the *gyrA* gene: 7 isolates with a Ser83-Phe substitution, 1 isolate (ST39) with an Asp87-Asn substitution, 1 isolate (ST24) with a Ser83-Tyr substitution, and 2 isolates (ST45 and ST104) with double amino acid substitutions Ser83-Phe and Asp87-Asn. ST45 and ST104 were found to be fully CIP resistant by disc diffusion with MICs of 8 and 4 mg/L, respectively. For the QRDR of the *parC* gene, the *S.* Typhi ST45 isolate was found to have a single amino acid substitution in the QRDR of the *parC* gene, whilst in the remaining isolates, including ST104, no mutation was detected (Table 3). Our results confirm the importance of the *gyrA* gene in fluoroquinolone resistance and the relevance of single and double mutations in this gene in controlling the degree of resistance to fluoroquinolones (16,18–20). Among *S.* Typhi with a single amino acid substitution, we observed the occurrence of various MICs of CIP in NA-resistant isolates. No mutation was detected in the QRDR-coding region of the *parC* gene, even in the *S.* Typhi ST104 isolate (high CIP resistance, MIC of 4 mg/L). This suggests that some other mechanisms, such as the increased expression of the efflux pump, in addition to *gyrA* and *parC* gene mutations, may contribute to the fluoroquinolone resistance of these isolates.

In conclusion, the persistence of NA resistance in *S.* Typhi isolates constitutes a major problem in Nepal. There is a decreasing trend in the identification of MDR isolates and this may imply the need to reevaluate the drugs of choice for the treatment of typhoid fever. NA resistance testing by disc diffusion can be used as a screen for *S.* Typhi with decreases CIP susceptibility.

Acknowledgments Acharya D. was supported by the Neighboring Countries International Scholarship; Korbsrisate *S.* was supported by the Chalermphrakiat Grant, Faculty of Medicine Siriraj Hospital, Mahidol University.

The authors are grateful to Ms. P. Tishyadhigama (NIH, Thailand) for her kind suggestions, Dr. J. Cuccui (LSHTM, UK) for editing the manuscript, and the Department of Microbiology, Dhulikhel Hospital for providing research facilities.

Conflict of interest None to declare.

Table 3. MICs of NA and CIP and nucleotide changes in QRDR of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) genes in clinical isolates of *S.* Typhi

Isolate	MIC (mg/L)		*gyrA* gene		*parC* gene	
	NA	CIP	Nucleotide change(s)	Amino acid change(s)	Nucleotide change(s)	Amino acid change(s)
ST 45	>256	8	TCC→TTC	Ser-83-Phe	AGC→ATC	Ser-80-Ile
			GAC→AAC	Asp-87-Asn		
ST104	>256	4	TCC→TTC	Ser-83-Phe	—	—
			GAC→AAC	Asp-87-Asn		
ST1	>256	0.5	TCC→TTC	Ser-83-Phe	—	—
ST36	>256	0.5	TCC→TTC	Ser-83-Phe	—	—
ST24	128	0.25	TCC→TAC	Ser-83-Tyr	—	—
ST26	256	0.25	TCC→TTC	Ser-83-Phe	—	—
ST39	128	0.25	GAC→AAC	Asp-87-Asn	—	—
ST66	256	0.25	TCC→TTC	Ser-83-Phe	—	—
ST100	256	0.25	TCC→TTC	Ser-83-Phe	—	—
ST103	256	0.25	TCC→TTC	Ser-83-Phe	—	—
ST8	128	0.125	TCC→TTC	Ser-83-Phe	—	—
ST77 (control)	8	0.06	—	—	—	—
ST79 (control)	8	0.06	—	—	—	—

— indicates no mutation.

NA, nalidixic acid; CIP, ciprofloxacin; QRDR, quinolone resistance-determining region.

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