RESEARCH NOTE

EMERGENCE AND PROPERTIES OF FLUOROQUINOLONE RESISTANT *SALMONELLA ENTERICA* SEROVAR TYPHI STRAINS ISOLATED FROM NEPAL IN 2002 AND 2003

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Abstract. A total of 171 *Salmonella enterica* serovar Typhi strains isolated from Nepal, mostly from patients with typhoid fever in 2002-2003, were tested for antimicrobial susceptibility by disk diffusion assay. Selected *S. enterica* serovar Typhi isolates were tested for MICs by E-test for ceftriaxone, ciprofloxacin and ofloxacin. Mutations of DNA gyrase *gyr*A and *gyr*B and topoisomerase IV *par*C and *par*E were identified by sequencing of PCR amplicons. By disk diffusion assay, 75/171 *S. enterica* serovar Typhi isolates were resistant to nalidixic acid, ampicillin, choramphenicol, streptomycin, tetracycline, sulfisoxazole, and trimethroprim/sulfamethoxazoles. Multiple drug resistance to the 7 antimicrobials was most predominant among *S. enterica* serovar Typhi isolates in this study. Resistance to nalidixic acid was detected in 76/111 and 56/60 of total isolates collected in 2002 and 2003, respectively. Nalidixic acid-resistant isolates in 2002 and 2003 showed MIC range for ciprofloxacin of 0.125-0.250 mg/l. Nalidixic acid-resistant isolates contained point mutations in *gyr*A and *par*C but not *gyr*B and *par*E. The *gyr*A mutation of nalidixic acid-resistant isolates obtained in 2002 and 2003 had amino acid substitution at position 83 of Serine→Tyrosine and Serine→Phenylalanine, respectively. Two different mutations of *gyr*A were detected among nalidixic acid-resistant isolates. Thus it is necessary to monitor mutation in DNA topoisomerase associated with increases in quinolones resistance.

Key words: *Salmonella enterica* serovar Typhi, antimicrobial resistances, quinolone resistance, typhoid outbreak, Nepal

INTRODUCTION

Salmonella enterica serovar Typhi is an invasive human pathogen that causes the systemic disease typhoid fever, a common febrile disease in developing countries, especially in Asia. Due to the increasing resistance to antimicrobials currently used traditionally for therapy (ampicillin/amoxicillin, co-trimoxazole, and chloramphenicol), the use of fluoroquinolones and broad-spectrum cepharosporin for the treatment of these infections has become

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more commonplace (Bhan *et al*, 2005). Quinolone antibiotics, which include the fluoroquinolones, act by inhibiting topoisomerases, DNA gyrase and topoisomerase IV (Turner *et al*, 2006). DNA gyrase catalyzes negative supercoiling of bacterial DNA and is essential to the maintenance of DNA replication. Topoisomerase IV is involved in the segregation of replicated daughter chromosomes during DNA replication. Both of these enzymes are composed of four subunits: 2 A and 2 B subunits encoded by *gyr*A and *gyr*B genes for DNA gyrase and 2 *par*C and 2 *par*E gene products for topoisomerase IV (Hopkins *et al*, 2005).

In *Salmonella*, the resistance to quinolones is typically caused by mutations in *gyr*A that result in amino acid substitutions in GyrA. The commonly observed mutations have been at the Ser-83 position, often to Tyr, Phe or Ala, and at Asp-87, substituting to Asn, Gly or Tyr. Mutation of *par*C, which encodes the ParC subunit of topoisomerase IV, leads to amino acid changes Thr-57→Ser, with Thr-66→Ile or Ser-80→Arg being observed as occasional second substitution (Giraud *et al*, 1999; Walker *et al*, 2003; Eaves *et al*, 2004; Gaind *et al*, 2006; Turner *et al*, 2006; Chau *et al*, 2007). In addition, a single mutation of *gyr*A together with mutation of *par*C may increase the levels of resistance (Ling *et al*, 2003). Mutations in *gyr*B and *par*E have rarely been reported (Giraud *et al*, 1999; Hirose *et al*, 2002; Ling *et al*, 2003; Turner *et al*, 2006). Some studies reported that the quinolones resistance gene is located on plasmids. Plasmid-mediated quinolone resistance was first reported in clinical isolates of *Salmonella enterica* in the UK in 2007 (Hopkins *et al*, 2007). Therefore, it is important to understand the mechanisms that cause high level resistance in *S. enterica* serovar Typhi (Ling *et al*, 2003).

In this study, mutations in the gyrase and topoisomerase IV genes were analyzed. A mechanism of fluoroquinolone resistance and the prevalence of multiple antimicrobial resistances among *S. enterica* serovar Typhi isolated from Nepal were determined.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 171 *S. enterica* serovar Typhi isolates, mostly from patients with typhoid fever between 2002 and 2003 in Bharatpur and Kathmandu, Nepal, were confirmed at the Department of Enteric Diseases, AFRIMS, Bangkok, Thailand. One hundred and sixty-four isolates were from blood cultures, 3 from stool specimens and 4 from water. All *S. enterica* serovar Typhi isolates were kept at -70 ± 5ºC in glycerol medium. Prior to testing, one loop of *S. enterica* serovar Typhi isolate from frozen stock cultures was subcultured on MacConkey Agar (MAC) and grown overnight at 37 ± 5ºC.

Antimicrobial susceptibility testing

S. enterica serovar Typhi isolates were tested for susceptibility to antimicrobials by a standard disk diffusion technique (NCCLS, 2002). The antibiotic disks contained: azithromycin (AZM, 15 μg), nalidixic acid (NAL, 30 μg), ciprofloxacin (CIP, 5 μg), ampicillin (AMP, 10 μg), chloramphenicol (CHL, 30 μg), colistin (CL, 30 μg), gentamicin (GEN, 10 μg), kanamycin (KAN, 30 μg), neomycin (N, 30 μg), streptomycin (STR, 10 μg), sulfisoxazole (G, 250 μg), trimethoprim/sulfamethoxazone (SXT, 1.25/23.75 μg), tetracycline (TET, 30 μg), and ceftriaxone (CRO, 30 μg) (BBL Sensi-Disc; Becton Dickinson, NJ, USA). Susceptibility and resistance were defined according to the criteria suggested by the NCCLS guideline, 2002. MIC of

Table 1
Primers used for PCR amplification and sequencing of genes coding for quinolone resistance.

Primer	Primer sequence
STGYRA1	5′-TGTCCGAGATGGCCTGAAGC-3′
STGYRA12	5′-CGTTGATGACTTCCGTCAG-3′
STGYRB5	5′-AAGCGCGATGGCAAAGAAG-3′
STGYRB6	5′-AACGGTCTGCTCATCAGAAAGG-3′
STGYRB7	5′-GAAATGACCCGCCGTAAAGG-3′
STPARC1	5′-ATGAGCGATATGGCAGAGCG-3′
STPARC2	5′-TGACCGAGTTCGCTTAACAG-3′
STPARE1	5′-GACCGAGCTGTTCCTTGTGG-3′
STPARE2	5′-GCGTAACTGCATCGGGTTCA-3′

Table 2
Pattern of antimicrobial-resistance of *S. enterica* serovar Typhi isolates.

Resistance phenotype	No. of isolates (%)
NAL AMP CHL STR G SXT TET	75 (44)
NAL AMP CHL STR G SXT	1 (1)
NAL CHL STR G SXT TET	1 (1)
NAL	29 (17)
NAL AMP CHL G SXT	21 (12)
NAL CHL TET	3 (2)
NAL AMP	1 (1)
NAL TET	1 (1)
AMP CHL STR G SXT TET	1 (1)
STR G	1 (1)
STR	1 (1)
Susceptible	36 (21)

NAL, Nalidixic acid; AMP, Ampicillin; CHL, Chloramphenicol; STR, Streptomycin; G, Sulfisoxazole; SXT, sulfamethoxazole/ Trimethoprim; TET, Tetracyclin

ciprofloxacin was determined using E-test strips (AB Biodisk, Solna, Sweden), following the manufacturer's instructions. *Escherichia coli* 25922 (with known MICs) were used as control for potency of antibiotics.

Amplification of *gyr*A, *gyr*B, *par*C, and *par*E genes in quinolones resistance determining region (QRDR)

For amplification of QRDR of *gyr*A, *gyr*B, *par*C, and *par*E gene, bacterial DNA template was prepared by resuspending a loop of *S. enterica* serovar Typhi isolate from MAC agar plate in Milli-Q water (200 µl) and boiling for 10 minutes, followed by centrifugation at 15,600*g* for 2 minutes to obtain the supernatant. The *gyr*A, *gyr*B, *par*C, and *par*E genes were amplified by PCR using primer (given in Table 1) and thermal cycling conditions as previously described (Giraud *et al*, 1999). PCR products were purified using Qiaquick® Purification Kit (Qiagen, Valencia, CA).

DNA sequencing and data analysis

All purified PCR products were submitted for sequencing by Macrogen (Macrogen, Seoul, Korea). Sequencer software version 4.6 (Gene Codes Corporation, Ann Arbor, MI) was used for sequence assembly. The sequences of QRDRs were determined between amino acids 54 and 171 of GyrA, 397 and 520 of GyrB, 12 and 130 of ParC, and 421 and 524 of ParE by Molecular Evolution Genetics Analysis

Table 3
Mutations in QRDR of DNA gyrase and topoisomserase IV subunits and MICs of ciprofloxacin in *S. enterica* serovar Typhi isolates collected in 2002 and 2003.

Year	Mutation in QRDRs				No. of isolates	Range of MIC (mg/l)
	GyrA	GyrB	ParC	ParE		CIP
2002	-	-	-	-	35 (23)	0.004-0.008
	Ser-83→Phe (TCC→TTC)	-	-	-	11 (7)	0.125-0.190
	Ser-83→Tyr (TCC→TAC)	-	-	-	64 (59)	0.125-0.190
	Ser-83→Tyr (TCC→TAC)	-	Gly78→Asp (GGC→GAC)	-	1 (1)	0.250
2003	-	-	-	-	4 (4)	ND
	Ser-83→Phe (TCC→TTC)	-	-	-	52 (13)	0.094-0.190
	Ser-83→Tyr (TCC→TAC)	-	-	-	4 (2)	0.125-0.190

ND, not determined
Number in parentheses indicates no. of tested *S. enterica* serovar Typhi isolates.

(MEGA) version 3.1 (Kumar *et al*, 2004; Tamura *et al*, 2004).

RESULTS

Antimicrobial susceptibility of all *S. enterica* serovar Typhi isolates is shown in Table 2. The most common antimicrobial resistance pattern of the isolates by disc diffusion assay was resistance to AMP, CHL, NAL, G, STR, SXT, and TET. This pattern accounted for 75 of the 171 (44%) *S. enterica* serovar Typhi isolates. The amplified PCR products of QRDR of *gyr*A, *gyr*B, *par*C, and *par*E were sequenced and analyzed. One hundred thirty-two of NAL-resistant isolates contained a mutation that encoded an amino acid substitution within QRDR of *gyr*A. Of these 132 isolates, 99% (131/132) contained single mutations in *gyr*A (Ser83→Phe or Ser83→Tyr) and 1% (1/132) contained mutations in *gyr*A and *par*C (Ser83→Tyr in *gyr*A and Gly78→Asp in *par*C) (Fig 1). The selected *S. enterica* serovar Typhi isolates were subjected to ciprofloxacin MIC determination by E-test (summarized in Table 3).

DISCUSSION

In this study, the predominant antimicrobial resistance pattern was resistance to AMP, CHL, NAL, G, STR, SXT, and TET. A single mutation within QRDR of *gyr*A can be sufficient to cause resistance to nalidixic acid, but an additional mutation may be required to attain high-level fluoroquinolone resistance (Wain *et al*, 1997; Piddock *et al*, 1998; Cloeckaert and Chaslus-Dancla, 2001). As demonstrated in Fig 1, the *gyr*A mutations of NAL-resistant isolates found in this study (Ser83→Tyr and Ser83→Phe) have been reported previously (Hopkins *et al*, 2005). The *gyr*A mutation changed from Ser83→Tyr in 2002 to Ser83→Phe in 2003. The NAL-resistant isolates containing Ser-83→Tyr showed a narrow MIC range for CIP (0.125-0.190 mg/l). The NAL-resistant

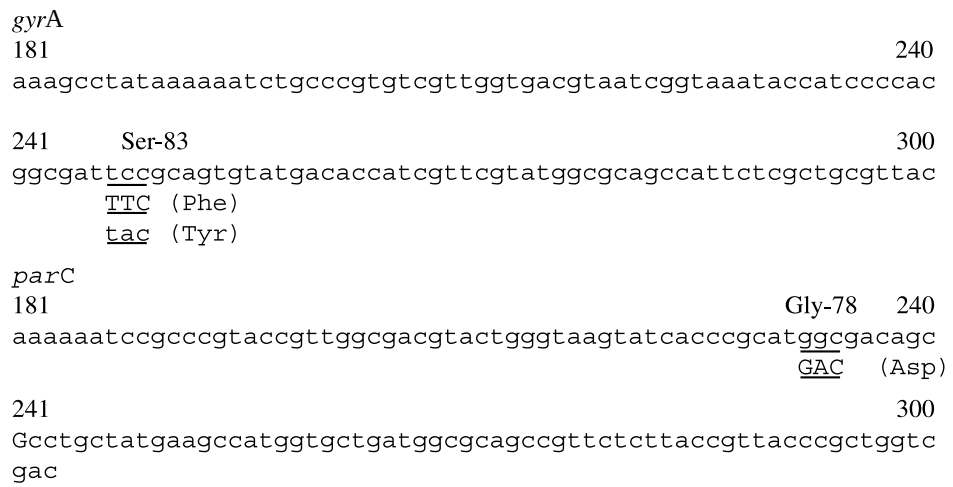

Fig 1–Base sequence of QRDR of *gyr*A and *par*C of *S. entrica* serovar Typhi isolates. The triplet bases with changes found in this study are underlined with the amino acids indicated above them. The underlined triplet bases in lowercase letters are found in *S. enterica* serovar Typhi isolates from 2002, and those underlined in capital letters are found in 2003.

isolates containing mutation Ser-83→Phe showed a wider MIC range for CIP (0.094-0.190 mg/l). As shown in Table 3, CIP MICs for the susceptible isolates were ≤ 0.008 mg/l. Generally, ciprofloxacin MIC used to define resistant isolates was ≥ 4 mg/l. In this study, the ciprofloxacin MICs for *S. enterica* serovar Typhi isolates with a single mutation at codon 83 of *gyr*A (Ser→Phe or Tyr) were between 12-fold (0.094/0.008) and 48-fold (0.190/0.004) higher than those of isolates with no mutation. Mutation in the QRDR of the *gyr*A gene, especially at position 83, is a common site for fluoroquinolone resistance as seen in our results.

Some previous studies have reported that the residues associated with mutations leading to high-level quinolone resistance contained a single mutation in *par*C in addition to a double mutation in *gyr*A (Eaves *et al*, 2004; Gaind *et al*, 2006). In this study, only 1 of the 171 isolates contained a single mutation *par*C at position Gly-78→Asp together with a mutation of *gyr*A at position Ser 83→Tyr, and the isolate had a higher CIP MIC. Mutation in *par*C also led to high level quinolone resistance when found in addition to mutation of *gyr*A. The CIP MIC for this isolate (0.250 mg/l) was about 2-fold higher than those with a single *gyr*A mutation (Ser→Phe or Tyr).

In *S. enterica* serovar Typhi, the primary target of fluoroquinolones is DNA gyrase rather than topoisomerase IV; hence mutation of *gyr*A precedes that of *par*C (Ling *et al*, 2003; Gaind *et al*, 2006). No point mutations in *gyr*B and *par*E genes were found in this study. This is not surprising as *gyr*B and *par*E mutations remain extremely rare in most bacterial species, even among highly resistant strains. It has been suggested that *gyr*B and *par*E mutations may not play an important role in NAL resistance (Ling *et al*, 2003).

In summary, this study has provided evidence that multiple drug resistance to 7 antimicrobials was the most predominant resistant phenotype among *S. enterica*

serovar Typhi isolates in Nepal. Single amino acid substitutions in *gyr*A were the mechanism of NAL resistance and reduced susceptibility to fluoroquinolones, while an additional with *par*C mutation increased the resistance to a higher level. It may be necessary to monitor the incidence of *gyr*A and *par*C mutations associated with an increase of fluoroquinolone resistance level. In order to find out whether fluoroquinolone resistance associated with *S. enterica* serovar Typhi isolates is located on transformable plasmids, transformation experiments and plasmid analysis should be performed.

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