Original Article

Molecular Analysis of Ciprofloxacin Resistance among Non-Typhoidal *Salmonella* with Reduced Susceptibility to Ciprofloxacin Isolated from Patients at a Tertiary Care Hospital in Kuala Lumpur, Malaysia

Rina Karunakaran^{1*}, Sun Tee Tay¹, Fairuz Fadzilah Rahim¹,
Bee Bee Lim¹, and Savithri D. Puthucherry^{1,2}

¹Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; and

²Medical Education, Research and Evaluation Department, Duke-NUS Graduate Medical School Singapore, 8 College Road, Singapore

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SUMMARY: We investigated the prevalence of non-typhoidal *Salmonella* (NTS) with “reduced susceptibility to ciprofloxacin” (RS-Cip) (minimum inhibitory concentration [MIC], 0.12–1.0 µg/mL) as well as their resistance genes in 75 NTS isolates (53 from stool, 21 from blood, and 1 from urine) from patients at a tertiary care Malaysian hospital between January and December 2009. RS-Cip was detected in 24/75 (32.0%) isolates. Using the ciprofloxacin MIC interpretive criteria for *Salmonella* in the Clinical and Laboratory Standards Institute 2013 guidelines, 51/75 (68.0%) isolates were found to be sensitive, 22/75 (29.3%) were intermediate, and 2/75 (2.7%) were resistant to ciprofloxacin. The 24 isolates that were intermediate or resistant to ciprofloxacin were the same isolates categorized as having RS-Cip. Among the 23 tested isolates with RS-Cip, the *qnrS* gene was detected in 17/23 (73.9%) and single *gyrA* mutations were detected in 6/23 (26.1%) (Asp87Tyr [*n* = 3], Asp87Asn [*n* = 2], and Ser83Phe [*n* = 1]). A *parC* (Thr57Ser) mutation was detected in 13/23 (56.5%) isolates, coexisting with either a *qnrS* gene or a *gyrA* mutation. The high incidence of the *qnrS* gene among isolates with RS-Cip needs to be monitored because *qnr* genes can spread via plasmids and aid in the emergence of increased resistance levels.

INTRODUCTION

Ciprofloxacin (a fluoroquinolone) is one of the important choices for the treatment of invasive, systemic, and extra-intestinal non-typhoidal *Salmonella* (NTS) infections in adults (1). However, antibiotic treatment of NTS gastroenteritis is not routinely recommended unless it is severe or occurring in patients having any of the risk factors listed in guidelines (2), with ciprofloxacin being one of the recommended antibiotics for adults (1,2).

As per the earlier Clinical and Laboratory Standards Institute (CLSI) guidelines (3), resistance to fluoroquinolones refers to isolates with a ciprofloxacin minimum inhibitory concentration (MIC) ≥ 4 µg/mL, and it has been referred to as “high-level resistance to fluoroquinolones” (4). *Salmonella* isolates were previously described as having decreased susceptibility to fluoroquinolones when MIC of ciprofloxacin was 0.12–1.0 µg/mL (4), and although these isolates are interpreted as “sensitive” by previous CLSI guidelines (3), they are of concern because therapeutic failures may result when a fluoroquinolone is used for the treatment of such in-

fection (5–11). The current CLSI 2013 guidelines (12) have revised ciprofloxacin interpretation for all *Salmonella* strains as follows: sensitive, ≤ 0.06 µg/mL; resistant, ≥ 1 µg/mL.

Fluoroquinolone resistance is usually caused by changes in the target enzymes DNA gyrase (subunits GyrA and GyrB) and topoisomerase IV (subunits ParC and ParE) and by changes in drug entry and efflux (13). One mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene in *Salmonella* usually results in concurrent resistance to nalidixic acid and reduced ciprofloxacin susceptibility (14–16). Additional mutations can further increase fluoroquinolone resistance (13,17). Plasmid-mediated quinolone resistance (PMQR) by the *qnr* gene, which was first described in *Klebsiella pneumoniae* (18), has also emerged recently (e.g., *qnrA*, *qnrS*, *qnrB*, *qnrC*, *qnrD*) (18–23). Other PMQR genes described include *aac(6′)-Ib-cr*, which causes resistance to aminoglycosides and reduced susceptibility to ciprofloxacin (24), *qepA* (encoding a fluoroquinolone efflux pump) (25), and *oqxAB* genes (encoding the multidrug efflux pump OqxAB) (26,27). PMQR results in low-level resistance to quinolones and aids in the emergence of increased resistance levels in the presence of therapeutic quinolone levels (19,28).

NTS isolates with reduced susceptibility to ciprofloxacin have been previously associated with resistance to nalidixic acid and a *gyrA* mutation (14). Older CLSI (National Committee for Clinical Laboratory Stand-

*Corresponding author: Mailing address: Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia. Tel: +60 3 79676661, Fax: +60 3 79676660, E-mail: rina@ummc.edu.my, rina_k@um.edu.my

ards) guidelines from 2004 (29) recommended testing extraintestinal isolates of *Salmonella* for resistance to nalidixic acid, because isolates that are sensitive to fluoroquinolones but resistant to nalidixic acid may not be eliminated by treatment with a fluoroquinolone (29). However, with the emergence of NTS isolates with reduced susceptibility to ciprofloxacin, which are either sensitive or have low-level resistance to nalidixic acid, researchers have cautioned that nalidixic acid screening may not identify all isolates with reduced fluoroquinolone susceptibility (30,32,33). This type of resistance phenotype has been reported in isolates without common *gyrA* mutations but with *qnr* genes (33–36). Current CLSI 2013 guidelines (12) advise that nalidixic acid may not detect all fluoroquinolone resistance mechanisms (12), and the revised ciprofloxacin disc diffusion and MIC interpretive criteria for all *Salmonella* strains enable direct testing of ciprofloxacin to determine the susceptibility of *Salmonella* to ciprofloxacin. Humphries et al. (4) have supported the implementation of the new CLSI ciprofloxacin breakpoints for all *Salmonella* strains and discussed the technical issues involved.

The National Surveillance of Antimicrobial Resistance from the Ministry of Health, Malaysia (2011) involving 36 hospitals (excluding our hospital) reported a ciprofloxacin resistance rate of 1.8% among *Salmonella* spp. (37). An earlier study (38) involving clinical *Salmonella* isolates from our hospital (2007–2008) reported a ciprofloxacin resistance rate of 1.3% and nalidixic acid resistance rate of 35.9%. On the other hand, a study from another tertiary hospital in Malaysia (39) reported that among the *Salmonella* isolates studied, 3.8% were resistant to ciprofloxacin by the disc diffusion method and 3.8% were intermediate, whereas 27.5% were resistant and 12.5% were intermediate to nalidixic acid. However, ciprofloxacin resistance rates are not directly comparable with current studies because of the revised CLSI ciprofloxacin interpretive criteria for *Salmonella* (12).

The present study aimed to (i) establish the prevalence of decreased (or reduced) susceptibility to ciprofloxacin (MIC, 0.12–1.0 µg/mL), as described in other reports (4), among NTS from patients at the University Malaya Medical Centre (UMMC) between January 2009 and December 2009 and (ii) detect possible mutations in *gyrA*, *gyrB*, *parC*, and *parE* and the presence of *qnr* genes among isolates with reduced susceptibility to ciprofloxacin. This ciprofloxacin MIC range (0.12–1.0 µg/mL) was previously interpreted as “sensitive” using the CLSI 2009 guidelines (3) but is now interpreted as “intermediate” (MIC, 0.12–0.5 µg/mL) or “resistant” (MIC, ≥ 1.0 µg/mL) for *Salmonella* on the basis of current CLSI 2013 guidelines (12).

MATERIALS AND METHODS

Isolates: NTS species included all species of *Salmonella*, except *Salmonella* Typhi and *Salmonella* Paratyphi. *Salmonella* spp. were identified from clinical samples by the Diagnostic Microbiology Laboratory, UMMC, on the basis of routine biochemical tests (40) and a limited range of *Salmonella* antisera for differentiating *S. Typhi* and *S. Paratyphi* from other NTS species. The API 20E system (bioMérieux SA, Marcy

l’Etoile, France) was occasionally used for further confirmation. The isolates were routinely stocked at –80°C in glycerol broth. All non-duplicate NTS isolates stocked from January to December 2009 were included in the present study. Only 1 isolate per patient was included, and when NTS was isolated from blood and a different site, only the blood isolate was included, with the exception of 2 cases where the blood isolate was not available.

Antimicrobial susceptibility: MICs of ciprofloxacin and nalidixic acid were determined using Etest (AB bioMérieux, Solna, Sweden) following the manufacturer’s recommendations. The isolates were categorized as having reduced or decreased susceptibility to ciprofloxacin if MIC of ciprofloxacin was 0.12–1.0 µg/mL (4), and this ciprofloxacin MIC range will be subsequently referred to as “reduced susceptibility to ciprofloxacin” (RS-Cip). Our study involved isolates from 2009, and the MIC interpretive criteria for ciprofloxacin were based on the CLSI 2009 guidelines (3) (sensitive, ≤ 1 µg/mL; intermediate, 2 µg/mL; and resistant, ≥ 4 µg/mL). However, because the ciprofloxacin MIC interpretive criteria for *Salmonella* are currently based on the CLSI 2013 guidelines (12) (sensitive, ≤ 0.06 µg/mL; intermediate, 0.12–0.5 µg/mL; and resistant, ≥ 1 µg/mL), MIC results were re-interpreted using this criteria, and for this report, only interpretations based on the CLSI 2013 guidelines (12) were used. MIC of nalidixic acid was interpreted on the basis of the CLSI guidelines (sensitive, ≤ 16 µg/mL; resistant, ≥ 32 µg/mL) (3,12).

For isolates with RS-Cip and for isolates with ciprofloxacin MIC of > 1.0 µg/mL, MICs of ciprofloxacin and nalidixic acid using Etest were determined 3 times (3 MIC determinations each). Isolates having ciprofloxacin MICs of 0.12–1.0 µg/mL in any of the MIC determinations were included in the category of RS-Cip for calculations.

Pseudomonas aeruginosa ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922 were used as quality controls for ciprofloxacin MIC testing, and *E. coli* ATCC 25922 was used as quality control for nalidixic acid MIC testing. For isolates with RS-Cip, susceptibility to ceftriaxone by disc diffusion (previously performed by the Diagnostic Microbiology Laboratory, UMMC, using the CLSI 2009 guidelines [3]) was retrieved from the laboratory information system and records.

Amplification and sequence analysis of ciprofloxacin resistance genes in isolates with Rs-Cip: Polymerase chain reaction (PCR) assays were performed on isolates with RS-Cip for the amplification of QRDR of *gyrA*, *gyrB*, *parC*, and *parE*, as described previously (41). Boiled bacterial extracts were used as DNA templates, and amplicons were purified using the PCR Purification Kit (Gene All Biotechnology, Seoul, Korea). Sequencing was performed using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems) using forward and reverse primers for each PCR assay. The obtained sequences were imported into the BioEdit sequence alignment program, version 7.0.5.3 (42), and inspected for the presence of mutation. A multiplex PCR assay was per-

formed for the simultaneous detection of *qnrA*, *qnrB*, and *qnrS*, as described previously (43). Upon agarose gel electrophoresis, the expected size of the amplified fragments for *qnrA*, *qnrB*, and *qnrS* genes was 580, 264, and 428 bp, respectively.

RESULTS

There were 75 isolates included in the study, of which 53 were from stool, 21 from blood, and 1 from urine.

Antimicrobial susceptibility: RS-Cip was present in 24/75 (32.0%) isolates. Based on the CLSI 2013 guidelines (12), 51/75 (68.0%) isolates were sensitive, 2 (2.7%) were resistant, and 22 (29.3%) were intermediate to ciprofloxacin (Table 1). The 24 isolates that were either intermediate or resistant to ciprofloxacin were the same isolates categorized as having RS-Cip. Nalidixic acid MIC results for isolates with RS-Cip are shown in Table 1.

Molecular analysis was performed on 23 of the 24 isolates with RS-Cip. Single *gyrA* mutations were detected in only 6/23 (26.1%) isolates, of which 3 were Asp87Tyr, 2 were Asp87Asn, and 1 was Ser83Phe (Table 1). Two of the 6 isolates also had an additional

parC (Thr57Ser) mutation. All 6 isolates with the *gyrA* mutation were resistant to nalidixic acid, with MIC of ≥ 256 $\mu\text{g/mL}$ repeatedly, and MIC of ciprofloxacin ranged from 0.094 $\mu\text{g/mL}$ to 0.19 $\mu\text{g/mL}$. A *parC* (Thr57Ser) mutation was detected in 13/23 (56.5%) isolates, which was associated with a *gyrA* mutation in 2 isolates (Asp87Asn and Ser83Phe) and a *qnr* gene in 11 isolates (Table 1). No *gyrB* or *parE* mutation was detected in the 23 isolates with RS-Cip.

All 17/23 isolates with RS-Cip but without a *gyrA* mutation had a *qnr* gene detected (Table 1). The only *qnr* gene detected from multiplex PCR analysis was *qnrS* in 17/23 (73.9%) isolates with RS-Cip, and among these *qnr*-positive isolates, *parC* (Thr57Ser) mutations were detected in 11/17 (64.7%) isolates. *qnrA* and *qnrB* were not detected in the isolates. MICs of ciprofloxacin for these 17 isolates ranged from 0.19 $\mu\text{g/mL}$ to 1.5 $\mu\text{g/mL}$ (intermediate to resistant), while MICs of nalidixic acid for these 17 isolates ranged from sensitive (5 isolates) to resistant (7 isolates), with varying between sensitive and resistant on repeated testing (5 isolates). We experienced some difficulty in reading MIC of nalidixic acid using Etest for many isolates having a *qnr* gene because it was sometimes subjective owing to the

Table 1. MICs of ciprofloxacin and nalidixic acid: interpretation and molecular detection of *gyrA/parC* mutation and *qnr* genes among 24 isolates with RS-Cip¹⁾

No./source	Serogroup	Ciprofloxacin		Nalidixic acid		<i>gyrA/parC</i> mutation	<i>qnr</i> gene
		MIC	int	MIC	int		
		Etest range ($\mu\text{g/mL}$) ²⁾	CLSI 2013 ³⁾	Etest range ($\mu\text{g/mL}$) ²⁾	CLSI 2013 ³⁾		
1/Bld	D*	0.094–0.19	I	≥ 256	R	<i>gyrA</i> (Asp87Tyr)	ND
2/Stl	C	0.25–0.38	I	12–16	S	ND	<i>qnrS</i>
3/Stl	C	0.38–0.5	I	24–48	R	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
4/Stl	C	0.38–0.5	I	32–96	R	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
5/Stl	B	0.25–0.38	I	12–24	S-R	ND	<i>qnrS</i>
6/Stl	C	0.19–0.25	I	12–16	S	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
7/Stl	B**	0.25–0.38	I	8–24	S-R	ND	<i>qnrS</i>
8/Stl	B	0.19–0.38	I	8–32	S-R	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
9/Stl	B**	0.25–0.38	I	16–24	S-R	ND	<i>qnrS</i>
10/Bld	D*	0.094–0.125	I	≥ 256	R	<i>gyrA</i> (Asp87Tyr)	ND
11/Bld	C	0.125	I	≥ 256	R	<i>gyrA</i> (Asp87Asn) <i>parC</i> (Thr57Ser)	ND
12/Stl	C	0.19–0.25	I	12–16	S	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
13/Stl	C	0.19–0.25	I	8–16	S	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
14/Stl	E	0.75–1	R	96– ≥ 256	R	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
15/Stl	C	0.38–0.5	I	24–48	R	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
16/Stl	C	0.38–0.5	I	24–48	R	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
17/Stl	C	0.25–0.38	I	8–12	S	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
18/Bld	C	0.75–1.5	R	192– ≥ 256	R	ND	<i>qnrS</i>
19/Stl	B	0.19–0.125	I	16–24	S-R	NT	NT
20/Stl	D	0.125	I	≥ 256	R	<i>gyrA</i> (Asp87Asn)	ND
21/Bld	D*	0.125	I	≥ 256	R	<i>gyrA</i> (Asp87Tyr)	ND
22/Stl	B**	0.25–0.5	I	32–48	R	ND	<i>qnrS</i>
23/Stl	C	0.25	I	16–24	S-R	<i>parC</i> (Thr57Ser)	<i>qnrS</i>
24/Stl	C	0.094	I	≥ 256	R	<i>gyrA</i> (Ser83Phe) <i>parC</i> (Thr57Ser)	ND

¹⁾ RS-Cip, reduced susceptibility to ciprofloxacin (MIC: 0.12–1.0 $\mu\text{g/mL}$; reference no. 4).

²⁾ MIC was performed 3 times.

³⁾ reference no. 12.

*, further identified as *S. Enteritidis*; **, further identified as *S. Typhimurium*.

MIC, minimum inhibitory concentration; int, interpretation; Bld, blood; Stl, stool; I, intermediate; S, sensitive; R, resistant; ND, not detected; NT, not tested.

presence of small isolated/single colonies within the Etest ellipse; these colonies were included when reading MIC unless they were far away from the Etest strip, in which case they were excluded. In these situations, Etests were repeated for verification. The reading of MIC of nalidixic acid using Etest in isolates with a *gyrA* mutation was easy, with MICs clearly being ≥ 256 $\mu\text{g/mL}$. None of the isolates with RS-Cip were also found to be resistant to ceftriaxone from previous laboratory records.

DISCUSSION

A study from Asia (44) during 2003–2005, which included NTS isolates from 7 countries (but not Malaysia), revealed that rates of RS-Cip (defined in that study as MIC, 0.125–1 $\mu\text{g/mL}$) were high in Taiwan (48.1%), Thailand (46.2%), and Korea (36.5%), and comparatively lower in Singapore (24.5%), the Philippines (14.9%), Hong Kong (7.1%), and Sri Lanka (8.0%). The rate of RS-Cip in our present study (32.0%) was higher than that in Singapore but lower than the rates in Korea, Thailand, and Taiwan observed in that previous Asian study; however, our study involved isolates from 2009.

Mutations at codon Ser83 or Asp87 are known to be common sites of mutation in *gyrA*, resulting in quinolone resistance (16,30), and all *gyrA* mutations detected in the present study (Asp87Tyr, Asp87Asn, and Ser83Phe) have been reported previously (14,16,41).

The commonest resistance gene detected in the present study among the 23 tested isolates with RS-Cip (study isolates that were either intermediate or resistant to ciprofloxacin as per the CLSI 2013 guidelines [12]) was the *qnr* gene (all *qnrS*), found in 17/23 (73.9%), and more than half of the isolates (11/17, 64.7%) had an additional *parC* (Thr57Ser) mutation. However, the *parC* (Thr57Ser) mutation has not been previously linked to reduced ciprofloxacin susceptibility (31,33), and Gunell et al. have reported that it is “likely to be a polymorphism common in serovars other than Typhimurium” (33).

There have been numerous reports of plasmid-mediated *qnr* genes detected in *Salmonella* with decreased/reduced susceptibility to ciprofloxacin from around the world (31,33–35,45–52). Studies have reported that since 2003 in Finland, NTS isolates with reduced susceptibility to fluoroquinolone (referring to ciprofloxacin MIC ≥ 0.125 $\mu\text{g/mL}$), which were either susceptible or had only low-level resistance to nalidixic acid, had been isolated from Finnish travelers returning from Thailand or Malaysia (30,32,33). These isolates were referred to as the “nonclassical quinolone resistance phenotype” (32,33), and isolates that were tested did not have a *gyrA* mutation (30,33). Ten isolates with this nonclassical quinolone resistance phenotype were further investigated and were found to have *qnr* genes, which confer this phenotype (*qnrS* in all except 1 which had *qnrA*), although efflux may also play some role (33). In the present study, *qnrS* was the only *qnr* gene detected in 17/23 tested isolates with RS-Cip; however, we did not determine the *qnrS* variants (53). A future study to determine *qnrS* variants may be helpful for epidemiological investigations. A study from the

United States (51) reported that among NTS isolates with decreased susceptibility to ciprofloxacin (referring to ciprofloxacin MIC ≥ 0.25 mg/L) collected in 2004–2006, 17 (9.8%) had *qnr* genes, of which 11 had *qnrS*. The *qnrS* gene was also the commonest *qnr* gene detected among selected *Salmonella* isolates tested for *qnr* genes in reports from the United Kingdom (*qnrS*) (45) and Denmark (mainly *qnrS*) (48,53) and in another report involving selected *Salmonella enterica* Corvallis isolated in Denmark and Thailand (*qnrS*) (36).

In the present study, we found that not all isolates with RS-Cip and the *qnr* gene were resistant to nalidixic acid, as reported previously (31,33). However, all our 6 isolates with RS-Cip and a *gyrA* mutation were nalidixic acid resistant, as described by Hakanen et al. (14), with MIC ≥ 256 $\mu\text{g/mL}$. Rodríguez-Martínez et al. (19) have reported that although reduced susceptibility to ciprofloxacin with low-level resistance or sensitivity to nalidixic acid may sometimes be observed in isolates having PMQR genes, this phenotype is not always a reliable indicator of PMQR and that clinical isolates with these genes often show high-level quinolone resistance owing to other resistance mechanisms. In the present study as well, isolate No. 14 and No. 18, in which *qnrS* genes were detected (together with a *parC* [Thr57Ser] mutation in isolate No. 14), had relatively high MICs of nalidixic acid (Table 1), and MICs of ciprofloxacin for both the isolates were interpreted as resistant according to the CLSI 2013 guidelines (12). It is possible that these 2 isolates may also have had other quinolone resistance mechanisms, which were not investigated in our study, such as reduced membrane permeability/efflux/*qepA/oqxAB/qnrC/qnrD/aac(6')-Ib-cr*.

qnr genes (encoding Qnr protein [54]) have been associated with extended-spectrum beta-lactamase (ESBL) genes (43,55–57). However, none of our isolates with RS-Cip in the present study were found to be resistant to ceftriaxone from previous laboratory records and were not investigated for the presence of ESBL genes.

In summary, RS-Cip was present in 32.0% of the 75 tested NTS isolates from 2009. Based on the CLSI 2013 guidelines (12), 68.0% of the NTS isolates were sensitive, 29.3% were intermediate, and 2.7% resistant to ciprofloxacin. The *qnrS* gene was detected in 17/23 (73.9%) isolates with RS-Cip, while *gyrA* mutations were detected in 6/23 (26.1%). Although 13 isolates had a *parC* (Thr57Ser) mutation coexisting with either a single *gyrA* mutation or a *qnrS* gene, this *parC* mutation has not been previously linked with RS-Cip (31,33). *qnr* genes cause low-level quinolone resistance, facilitate the emergence of increased resistance levels, and may be spread by plasmids (18,19,28,33). Therefore, the high prevalence of *qnr* genes among our NTS isolates with RS-Cip requires monitoring, and continued surveillance of ciprofloxacin susceptibility is important.

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Conflict of interest None to declare.

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