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**Analysis of mechanisms involved in reduced susceptibility to ciprofloxacin in *Salmonella enterica* serotypes Typhi and Paratyphi A isolates from travellers to Southeast Asia**

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## ABSTRACT

Owing to multidrug resistance, quinolones and third-generation cephalosporins are currently used as key antibiotics to combat *Salmonella* organisms. Therapy failure due to reduced ciprofloxacin susceptibility has been reported in endemic areas, but also in imported disease. Different bacterial resistance mechanisms may result in reduced ciprofloxacin susceptibility. In this study, the presence and expression of different resistance mechanisms resulting in reduced minimum inhibitory concentrations (MICs) for ciprofloxacin were evaluated in 23 blood culture-derived *Salmonella enterica* serotypes Typhi and Paratyphi A organisms from ill-returned travellers to Asia. The presence of mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene as well as an activated efflux pump and plasmid-mediated quinolone resistance genes was determined. Resistance selection during therapy and the clonal relatedness of all isolates were established. Efflux pump inhibition did not appear to affect the MICs of ciprofloxacin and activity of the efflux pump appeared to be specific for nalidixic acid. Repeated exposure of the isolates to ciprofloxacin did not result in a significant increase in the MICs for ciprofloxacin. Repetitive sequence-based polymerase chain reaction (rep-PCR) profiles identified five different genotypes, but no correlation with resistance was observed. However, a significant relation was found with geographic region; reduced ciprofloxacin susceptibility was only found in travellers returning from India and Pakistan. All isolates with reduced ciprofloxacin susceptibility had a mutation at position 83 in the QRDR region of the *gyrA* gene. Plasmid-mediated quinolone resistance was not found. These findings confirm that the reduced ciprofloxacin MIC in *S. Typhi* and *S. Paratyphi A* is solely due to an amino acid substitution in the QRDR 'cluster' of the *gyrA* gene.

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## 1. Introduction

Resistance of *Salmonella* spp. to ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole has been described during the last 20 years. Owing to this multidrug resistance, quinolones and third-generation cephalosporins are currently in use as key antibiotics to combat these multiresistant organisms [1]. As a consequence of the use of quinolones and cephalosporins, there have been reports describing clinical treatment failure following administration of ciprofloxacin to patients with typhoid fever owing to reduced ciprofloxacin susceptibility [defined as a minimum inhibitory concentration (MIC) of 0.125–1 µg/mL]. This reduced susceptibility is found not only in endemic areas but also in developed countries such as the USA and UK, reflecting a global problem [2,3].

Bacterial resistance to quinolones in *Salmonella* spp. may result from different resistance mechanisms. Quinolone resistance is usually associated with mutations in the target site (DNA gyrase), most commonly in the quinolone resistance-determining region (QRDR) of the GyrA subunit, conferring resistance to single-step mutants [4]. On the other hand, reduced susceptibility to quinolones is mostly due to mechanisms such as active efflux via efflux pumps as well as alterations in the expression of outer membrane proteins [5]. Plasmid-mediated quinolone resistance has also emerged in non-typhoidal *Salmonella enterica* strains resulting in reduced ciprofloxacin susceptibility; this mechanism has not yet been observed in typhoidal *Salmonella* spp. Outer membrane protein-deficient *Salmonella* spp. have only been described incidentally, resulting in increased MICs (>200 µg/mL) for chloramphenicol [6].

Owing to increased travel to endemic areas as well as migrant workers originating from endemic countries, The Netherlands is also confronted with patients not responding to empirical therapy with ciprofloxacin. In this retrospective study, *S. enterica* serotypes Typhi and Paratyphi A isolates were systematically characterised with respect to the presence and expression of different mechanisms of resistance causing reduced susceptibility to fluoroquinolones in 23 consecutive patients (travellers and migrant workers) diagnosed at the Institute for Tropical Diseases of Harbour Hospital–Rotterdam (The Netherlands) with blood culture-derived *S. Typhi* or *S. Paratyphi A* infection.

## 2. Materials and methods

### 2.1. Bacterial isolates

In total, 23 *Salmonella* isolates (*S. Typhi* from 11 individuals and *S. Paratyphi A* from 12 individuals) were collected from consecutive patients attending the Institute for Tropical Diseases during the period January 2002 to August 2008. All isolates were derived from positive blood cultures. Isolates were initially identified biochemically, followed by confirmation using specific antisera. Serotype determination was performed at the National Institute for Public Health and the Environment (RIVM) (Bilthoven, The Netherlands).

### 2.2. Antimicrobial susceptibility

Susceptibility of the isolates was determined using the VITEK-2 system (bioMérieux sa, Marcy l'Etoile, France). MICs for nalidixic acid and ciprofloxacin were determined



by the microbroth dilution technique. All tests were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines where applicable. For in vitro detection of efflux pump expression, a two-step dilution series of the antibiotics nalidixic acid, ciprofloxacin and chloramphenicol was made to detect the MIC in the absence and presence of the efflux pump inhibitor (EPI) phenylalanine  $\beta$ -naphthylamide (PA $\beta$ N) (20  $\mu$ g/mL). A reduction of the initial MIC by  $\geq 3$ -step difference upon exposure to the EPI is an indication for the presence of an activated efflux pump [7].

### 2.3. Polymerase chain reaction (PCR) screening and sequence analysis

The QRDR of *gyrA* was amplified by PCR using the primers described by Kariuki et al. [8]. Products resulting from amplifications were subjected to sequencing using a 3100 ABI Prism Genetic Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Analysis and comparison of nucleotide and amino acid sequence data were carried out using MegAlign software (DNASTAR Inc., Madison, WI) and programs available from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Presence of the *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes was determined by PCR [9,10]. The presence of integrons was determined using the method described by Lévesque et al. [11].

### 2.4. Molecular typing

Clonal relatedness of all the isolates was established using the repetitive sequence-based PCR (rep-PCR) DiversiLab<sup>®</sup> Microbial Typing System (bioMérieux) according

to the manufacturer's instructions. Isolates with  $\geq 95\%$  identical profiles were considered to be closely related.

### 3. Results and discussion

During the last decade, treatment failures with ciprofloxacin have been increasingly reported. These failures have been associated with infection with *S. Typhi* and *S. Paratyphi A* strains that are resistant to nalidixic acid with decreased susceptibility to ciprofloxacin [2,3,12]. Studies describing mechanisms of fluoroquinolone resistance demonstrate only one or two specific mechanisms. However, several mechanisms of resistance can result in reduced susceptibility to ciprofloxacin [4]. Moreover, a combination of resistance mechanisms could also lead to enhancement of each of the separate mechanisms resulting in increased MICs. As mentioned previously, studies describing a systematic analysis of fluoroquinolone resistance mechanisms in different *S. Typhi* and *S. Paratyphi A* isolates are rare [13]. Therefore, we have systematically evaluated the presence and expression of different mechanisms of resistance resulting in reduced MICs for ciprofloxacin in 23 *Salmonella* clinical isolates.

Of the 23 isolates, 14 (60.9%) were resistant to nalidixic acid (NAL<sup>R</sup>) and displayed reduced MICs for ciprofloxacin ranging from 0.25  $\mu\text{g/mL}$  to 2  $\mu\text{g/mL}$ , except for one isolate (6838) that had a ciprofloxacin MIC of 32  $\mu\text{g/mL}$ . The remaining nine isolates (39.1%) were all nalidixic acid-susceptible (NAL<sup>S</sup>) and had low MICs for ciprofloxacin ( $\leq 0.03$   $\mu\text{g/mL}$ ) (Fig. 1). One of the mechanisms resulting in reduced susceptibility to quinolones may be the presence of activated efflux pumps. To detect the presence of

these pumps, a phenotypic method was applied by determining MICs for nalidixic acid and ciprofloxacin in the presence and absence of the EPI PA $\beta$ N. In most isolates, moderate efflux activity was determined for nalidixic acid resulting in MIC reductions of 3–4 steps in the presence of the EPI. However, the activity of this particular efflux pump(s) does not appear to affect the MICs of ciprofloxacin, indicating that the activity of this pump is specific, at least for nalidixic acid. As this phenomenon is not exclusively found in NAL<sup>R</sup> isolates, it is concluded that this specific efflux pump(s) is not causing the increased MICs for ciprofloxacin in the NAL<sup>R</sup> isolates (data not shown). Patients infected with isolates with reduced susceptibility to fluoroquinolones react less favourably on the initiated therapy [12]. This might be due to the fact that the strains that are already resistant to nalidixic acid may require fewer exposures to fluoroquinolones to develop high-level resistance to ciprofloxacin than strains that are fully ciprofloxacin-susceptible [5].

To check for resistance selection during therapy, a selected number of isolates were exposed for two rounds to increasing concentrations of ciprofloxacin. However, this limited exposure did not result in a significant increase in the MICs of ciprofloxacin. Also, efflux pump activity did not change after these two rounds of selection/induction by repeated exposure to ciprofloxacin (data not shown). In fact, the same base level efflux pump activity was observed for chloramphenicol (efflux indicator antibiotic) as was already demonstrated for nalidixic acid. Thus, the impaired clinical response is not due to resistance selection during antibiotic treatment but may be due to unfavourable pharmacodynamic parameters resulting in impaired killing.

Resistance or reduced susceptibility to fluoroquinolones is due the presence of active efflux pumps, impaired outer membrane permeability or gyrase mutations. As efflux does not explain the current findings, all isolates were subjected to sequencing of the *gyrA* gene. Mutations in the QRDR region of the *gyrA* gene resulting in amino acid substitutions are given in Fig. 1. All NAL<sup>S</sup> isolates show no mutations in the QRDR region. The NAL<sup>R</sup> isolates all had mutations at position 83 resulting in a substitution of serine to phenylalanine or tyrosine.

Isolate 6838, with a ciprofloxacin MIC of 32 µg/mL, for had a double mutation. In addition to the mutation at position 83, a substitution of asparagine to glycine at position 87 was also demonstrated. Mutations in *gyrA* outside of the QRDR were observed both in NAL<sup>R</sup> and NAL<sup>S</sup> isolates (data not shown). These data are in full agreement with the data of Nair et al. [14] who demonstrated that a single *gyrA* mutation at Ser-83 alone resulted in resistance to nalidixic acid and reduced susceptibility to ciprofloxacin (MICs of 0.125–0.25 µg/mL). Therefore, it was concluded that Ser-83 is an important site for determining fluoroquinolone resistance within *S. Typhi* and *S. Paratyphi A* isolates. Complete resistance to ciprofloxacin (MIC > 4 µg/mL) has been reported by Gaind et al. [13] and is due to a double mutation in the QRDR region.

In this investigation, the presence of plasmid-encoded *qnr* genes resulting in reduced susceptibility to quinolones in *Salmonella* spp. was also studied [15]. Expression of these genes is phenotypically hard to detect as they have a moderate effect on ciprofloxacin MICs even in combination with the *aac(6')-Ib-cr* gene. In the 23 isolates

studied, it was not possible to demonstrate the presence of plasmid-encoded *qnr* genes or *aac(6)-Ib-cr*, indicating that these *qnr*-bearing plasmids are 'host' specific. Therefore, these findings confirm other reports that the reduced MICs of ciprofloxacin in *S. Typhi* and *S. Paratyphi A* are solely due to the amino acid substitution in the QRDR 'cluster'. Besides reduced susceptibility to quinolones, two *S. Typhi* isolates demonstrated resistance to ampicillin, chloramphenicol, tetracycline and trimethoprim. One *S. Paratyphi A* isolate was resistant to ampicillin, chloramphenicol and tetracycline. In the two trimethoprim-resistant *S. Typhi* isolates an 800-bp integron cassette was demonstrated, indicating that the gene involved is probably the *dfrA7* gene encoding for a dihydrofolate reductase [16]. Integrons have been demonstrated in non-typhoidal *Salmonella* but only occasionally in *S. Typhi* [17].

To study the genetic relationship between the NAL<sup>R</sup> and NAL<sup>S</sup> clusters, all isolates were analysed using a commercial rep-PCR system. Results of rep-PCR (Fig. 1) showed three different clusters of 8, 2 and 11 isolates as well as 2 unique isolates. In clusters A and D consisting of 8 and 11 isolates, respectively, susceptibility to the quinolones was randomly distributed, thereby demonstrating that resistance to nalidixic acid, i.e. reduced ciprofloxacin susceptibility, is not linked to a distinct cluster. Isolates belonging to cluster A were all *S. Typhi* isolates, whereas cluster D comprised *S. Paratyphi A* isolates. The only consistent correlation found related to the country of acquisition of the infection, i.e. resistance to nalidixic acid and reduced susceptibility to ciprofloxacin was only found in travellers to India and Pakistan, but not in travellers to Indonesia and Cambodia. However, these geographical differences are not always that clear, as NAL<sup>R</sup> isolates have also been reported in Vietnam [2]. However, based on these data it seems at least indicated to treat

patients returning from India with an alternative antibiotic drug instead of ciprofloxacin.

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**Competing interests**

None declared.

**Ethical approval**

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**Fig. 1.** Genotyping of 23 isolates by repetitive sequence-based polymerase chain reaction (rep-PCR) (DiversiLab®). MIC, minimum inhibitory concentration; QRDR, quinolone resistance-determining region.

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