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Original Article

Characterization of non-classical quinolone resistance in *Salmonella enterica* serovar Typhi: Report of a novel mutation in *gyr*B gene and diagnostic challenges

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ABSTRACT

Objective: To establish the relative importance of *Salmonella enterica* serovar Typhi with non-classical quinolone resistance.

Methods: Eight hundred and ninety-one isolates of *S.* Typhi, isolated between 2004 and 2011, were tested for antibiotic susceptibility determination using disc diffusion and E-test. The mechanisms of fluoroquinolone resistance were studied in a sub-set of the NAL^S (nalidixic acid susceptible) isolates by wave nucleic acid fragment analysis of PCR products from *gyrA*, *gyrB*, *parC* and *parE* and from the plasmid borne determinants: *qnrA*,B,S; aac(6′)-Ib-cr and *qepA*. To assess genetic relatedness multi-locus variable number tandem repeat analysis was carried out using five loci.

Results: Eighty isolates with a nalidixic acid MIC of <32 mg/L (NAL^S) and a ciprofloxacin MIC of >0.064 mg/L CIP^I (ciprofloxacin reduced susceptibility) were found. In 36 NAL^S CIP^I isolates two distinct genotypes were identified when compared with 16 susceptible controls: Group B (n = 34), mutation in *gyrB* at codon 464, NAL MIC of 3–12 mg/L and CIP MIC of 0.064–0.5 mg/L.; and Group C, mutation in *gyrA* at codon 83 (n = 2) NAL MIC of 16 mg/L and CIP MIC of 0.25–0.38 mg/L. Group B isolates were found in different strain backgrounds as defined by MLVA.

Conclusion: The use of nalidixic acid to screen for reduced susceptibility to fluoroquinolones in *S.* Typhi misses CIP^I-NAL^S isolates, an established phenotype in India.

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1. Introduction

Typhoid fever, caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), remains a global public health problem centred in developing countries [1]. The emergence of multi-drug resistant (MDR) *S.* Typhi, with resistance to all first line drugs: (chloramphenicol, co-trimoxazole and ampicillin) led to the fluoroquinolones becoming the treatment of choice for enteric fever [2]. There is a clear relationship between increasing fluoroquinolone MIC and outcome during fluoroquinolone treatment of typhoid fever. Therefore for clinical management, any increase in fluoroquinolone MIC needs to be detected [3]. No zone around a nalidixic acid 30 μg disc has been used as a screening test for isolates with a clinically relevant ciprofloxacin resistance; MIC of ≥0.125 mg/L with a sensitivity of 92.9% (248/267); and a specificity of 98.4% (540/549) [4]. The most common mutation associated with reduced susceptibility to fluoroquinolones in *S.* Typhi is in the quinolone resistance determining region of the GyrA sub-unit of DNA gyrase. This mutation, at amino acid 83, also confers resistance to nalidixic acid (NAL^R) and so nalidixic acid can be a marker for fluoroquinolone resistance where this mutation is the sole cause of resistance. Strains of *S.* Typhi with decreased susceptibility to fluoroquinolones (e.g. ciprofloxacin (CIP^I) MIC > 0.064 mg/L are now very common in India [3]. The reports of alternative mechanisms of fluoroquinolone resistance are increasing [5,6]. In response to this the CLSI has redefined the breakpoints for ciprofloxacin as ≤0.064 mg/L and ≥1 mg/L and

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≥31 mm and ≤20 mm for susceptibility and resistance using MIC and disc diffusion [7], and strains that are >0.064 mg/L and <1 mg/L as intermediate for *Salmonella enterica*. Many laboratories in India however continue to test nalidixic acid as a marker for decreased susceptibility to fluoroquinolones (CIP^I) and so a formal report on the utility of the nalidixic acid screening test in India is of great importance to guide clinical management. Furthermore there is conflicting evidence on the role of fluoroquinolones mutations in clinical isolates; two mutations in *gyrA* and/or *gyrB* can be associated with full resistance to fluoroquinolones (MIC ≥ 4 mg/L) [8] but genetic manipulation of isogenic mutants shows that this is not the full story – other mutations must also be present [9]. It is therefore important to characterize circulating strain.

The Indian sub-continent reports more typhoid fever than any other world region and so monitoring of antibiotic resistance in *S.* Typhi in India can be used to detect any trend towards increasing ciprofloxacin resistance. Our centre is one of the busiest in India and surveillance of ciprofloxacin resistance has been carried out since 2001. In a previous study from 2003 the majority (282/285) of isolates with CIP^I exhibited the classical quinolone resistance phenotype, NAL^R-CIP^I and three isolates with NAL^S-CIP^I (CIP-MIC 0.125 mg/L) were observed [10]. Here we present data on the genetic and phenotypic characterization of CIP^I isolates of *S.* Typhi from 2004 to 2011.

2. Methods

All isolates were from patients admitted to the Safdarjung Hospital during 2004–2011 and were from blood or stool cultures, deduplication was carried out as far as possible using the clinical data available retrospectively. The Minimum Inhibitory Concentration (MIC) for nalidixic acid and ciprofloxacin against 891 *S.* Typhi was determined by E-test (AB Biodisk, Solna, Sweden). Results were interpreted by two microbiologists and discrepant results were repeated. Susceptibilities to nalidixic acid (30 μg), ciprofloxacin (5 μg) chloramphenicol (30 μg), co-trimoxazole (25 μg) and ampicillin (10 μg) were determined by disc diffusion (DD) test according to CLSI guidelines [7]. The breakpoints used for CIP^I were 0.064–1.0 mg/L and 21–30 mm for MIC and DD respectively. MDR was defined as resistance to chloramphenicol, co-trimoxazole, and ampicillin. The data was analyzed using WHONET 5.6. software.

Table 1
Primer sequences of plasmid mediated resistance (PMQR) genes: *qnr*A, *qnr*B, *qnr*S, *aac(6′)-Ib-cr* and *qep*A.

S. No.	Primer of PMQR genes	Amplicon size
1.	*qnr*A FP: 5′-ATT TCT CAC GCC AGG ATT TG-3′ RP: 5′-GAT CGG CAA AGG TTA GGT CA-3′	516 bp
2.	*qnr*B FP: 5′-GAT CGT GAA AGC CAG AAA GG-3′ RP: 5′-ACG ATG CCT GGT AGT TGT CC-3′	469 bp
3.	*qnr*S FP: 5′-ACG ACA TTC GTC AAC TGC AA-3′ RP: 5′-TAA ATT GGC ACC CTG TAG GC-3′	417 bp
4.	*aac(6′)-Ib-cr* FP: 5′-TTG CGA TGC TCT ATG AGT GGC TA-3′ RP: 5′-CTC GAA TGC CTG GCG TGT TT-3′	489 bp
5.	*qepA* FP: 5′-GCA GGT CCA GCA GCG GGT AG-3′ RP: 5′-CTT CCT GCC CGA GTA TCG TG-3′	1100 bp

From the NAL^S *S.* Typhi (MIC < 32 mg/L) 52 strains were selected to represent all years of isolation and all antibiograms to characterize the mechanism of FQ resistance. PCR was used to amplify the QRDRs of *gyr*A, *gyr*B, *par*C and *par*E genes [3]. Denaturing high-pressure liquid chromatography (DHPLC: Wave Nucleic Acid Fragment Analysis System; Transgenomic Inc.) was used to study mutations [11]. Mutations in the genes studied were identified by comparison with DNA sequences from *S.* Typhi strains Ty2 (GenBank accession no. AE014613). Screening for the plasmid determinants (*qnr*A,B,S; *aac(6′)-Ib-cr* and *qepA*) was carried out by PCR (primers in Table 1).

Multi-locus variable number tandem repeat (VNTR) analysis (MLVA) was carried out to assess genetic relatedness using five VNTR loci (TR1,TR2, TR4699, Sal02 and Sal16) [12]. Primers were tagged with 6′FAM (blue) fluorescent dye. Fragment length analysis was done by capillary electrophoresis (ABI3130 Genetic analyzer, Applied Biosystems). Fragment sizes were binned into alleles. Copy number was confirmed by sequencing. A cluster dendogram was constructed using R-software.

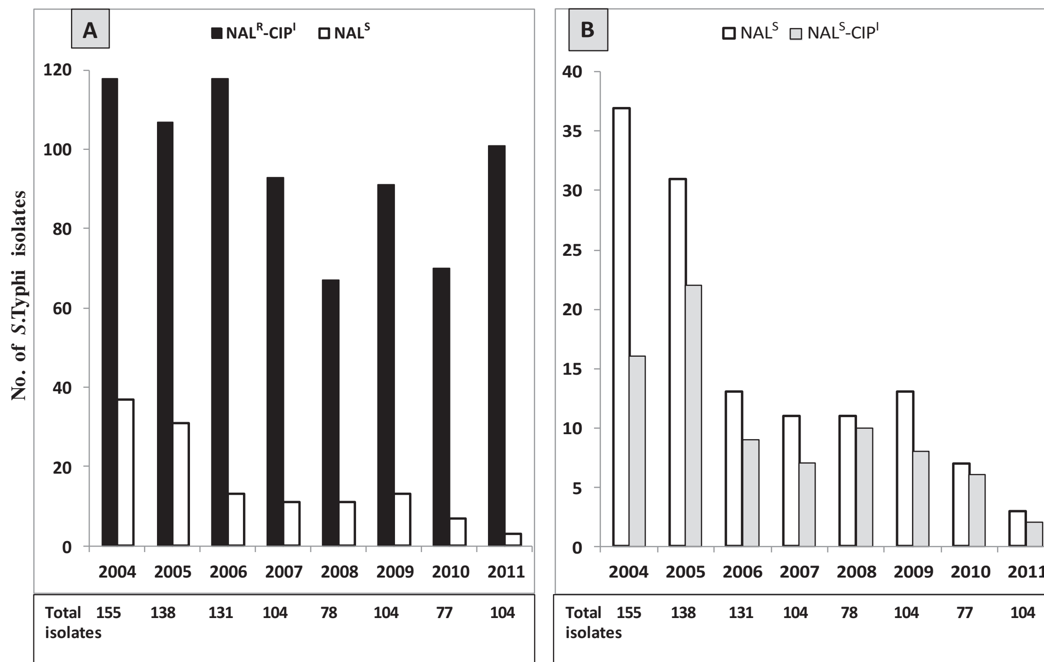

Fig. 1. The relative numbers of isolates of *Salmonella* Typhi at Safdarjung hospital. (A) NAL^S and NAL^R-CIP^I. (B) NAL^S and NAL^S-CIP^I. *Note*: NAL^S = nalidixic acid susceptible (MIC ≤ 16 mg/L). NAL^R = nalidixic acid resistance (MIC ≥ 32 mg/L). CIP^I = decreased ciprofloxacin susceptible (MIC > 0.064 mg/L).

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