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SHORT COMMUNICATION

Molecular Characterization of Intermediate Susceptible Typhoidal *Salmonella* to Ciprofloxacin, and its Impact

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Abstract

Background and Objective Extensive use of ciprofloxacin to treat *Salmonella* typhi infections has led to the emergence of resistance, resulting in clinical failure and delayed treatment response. Interpretative breakpoints for ciprofloxacin were revised by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in 2012. Since the majority of *S.* typhi isolates fall under the category of 'intermediate susceptible' as per CLSI criteria, we undertook molecular characterization to better define the susceptibility of these isolates.

Methods Of 113 typhoidal *Salmonella* isolates collected during 2014, 33 (27 *S.* typhi and 6 *S.* paratyphi A) were randomly selected to determine the presence of chromosomal (*gyr*A, *gyr*B and *par*C), plasmid (*qnr*A, *qnr*B, *qnr*S and *aac(6′)-lb-cr*), and efflux-mediated fluoroquinolone resistance.

Results To the best of our knowledge, the *par*C mutation Glu(84)-Gly was observed for the first time in *S.* typhi in India. Of 33 isolates, only one harbored the *qnr*B gene, which is responsible for plasmid-mediated resistance. No significant change in efflux pump activity was observed for ciprofloxacin, except one that showed a fivefold decrease. Ninety-six percent of isolates with intermediate minimum inhibitory concentration to ciprofloxacin (CLSI) had mutations in the *gyr*A and *par*C genes, which might translate to possible/probable clinical failure in patients if treated with ciprofloxacin. In contrast, the EUCAST criteria define these isolates as resistant and may result in appropriate therapy with reduced morbidity.

Conclusion It was clear that the molecular mechanism of ciprofloxacin resistance correlates better with the EUCAST criteria than the CLSI criteria, which is also in agreement with the pefloxacin results, suggesting it as a surrogate marker for identifying fluoroquinolone susceptibility.

Key Points

Ninety-six percent of typhoidal *Salmonella* isolates with intermediate minimum inhibitory concentration to ciprofloxacin (CLSI) had mutations in the *gyr*A and *par*C genes, which might translate to possible/probable clinical failure in patients if treated with ciprofloxacin.

The molecular mechanism of ciprofloxacin resistance correlates better with the EUCAST criteria than the CLSI criteria, which is also in agreement with the pefloxacin results, suggesting it as a surrogate marker for identifying fluoroquinolone susceptibility.

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1 Introduction

Multidrug-resistant (MDR) *Salmonella* typhi is defined as an isolate resistant to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole [1]. Ciprofloxacin was

recommended as a first-line therapy for MDR *S.* typhi and, over the past 15 years, MDR *S.* typhi has gradually decreased [2]. Meanwhile, extensive use of ciprofloxacin over this period has led to clinical failure and delayed treatment response [3, 4]. Reports from several regions of India have shown variable ciprofloxacin-resistant rates for *S.* typhi, ranging from 8 to 21.4 % [5–8].

Responding to this development in 2012, fluoroquinolone interpretative breakpoints for ciprofloxacin have been revised by the Clinical and Laboratory Standards Institute (CLSI), where the susceptibility cut-off using the disc diffusion method was raised from 21 to 31 mm, and the minimum inhibitory concentration (MIC) value was lowered from 1 to 0.06 μg/ml. Subsequently, in 2013 the disc diffusion interpretative criteria of levofloxacin and ofloxacin for *S.* typhi were removed, and the MIC interpretative criteria for levofloxacin and ofloxacin were lowered to ≤0.12 μg/ml (susceptible), 0.25–1 μg/ml (intermediate) and ≥2 μg/ml (resistant). It is noteworthy that the interpretative criteria for ciprofloxacin, levofloxacin and ofloxacin have only been changed for typhoidal *Salmonella* in the Enterobacteriaceae family.

After the aforementioned breakpoint revision, the vast majority (88 %) of *S.* typhi isolates were observed to be in the intermediate susceptibility to ciprofloxacin category [2], as per the CLSI criteria [9]. In the "intermediate" category, isolates may or may not respond to fluoroquinolone treatment. Clearly, the intermediate category is unreliable. In contrast, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) does not have an 'intermediate' category and defines the isolate as 'susceptible' or 'resistant' [10].

Since the vast majority of *S.* typhi isolates fall within the intermediate category, we undertook molecular characterization, and compared the results with CLSI and EUCAST criteria to better define the susceptibility of these isolates. Molecular characterization included determining the presence of chromosomal (*gyr*A, *gyr*B and *par*C), plasmid (*qnr*A, *qnr*B, *qnr*S and *aac(6′)-lb-cr*), and efflux-mediated fluoroquinolone resistance.

2 Materials and Methods

2.1 Typhoidal Salmonella Isolates

Of 113 typhoidal *Salmonella* isolates obtained from blood stream infections of patients at the Christian Medical College, Vellore (January to December 2014), 95 were *S.* typhi and 18 were *S.* paratyphi A. Of these, 33 isolates (which included 27 *S.* typhi and 6 *S.* paratyphi A) were randomly selected using a simple random sampling method for molecular characterization.

2.2 Antimicrobial Susceptibility Testing

2.2.1 Disc Diffusion

All isolates obtained were screened for antimicrobial susceptibility by the Kirby–Bauer method using ampicillin (10 μg), chloramphenicol (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg) and ceftriaxone (30 μg), according to guidelines suggested by CLSI 2014. Quality-control strains used were *Escherichia coli* ATCC 35218 for ampicillin and *E. coli* ATCC 25922 for the remaining antibiotics, concurrently in all the batches. Pefloxacin (5 μg) screening was carried out as per EUCAST 2014 and CLSI 2015 recommendations.

2.2.2 Minimum Inhibitory Concentration Testing

The ciprofloxacin MIC breakpoints defined in CLSI 2014 (≤0.06 μg/ml [susceptible], 0.12–0.5 μg/ml [intermediate] and ≥1 μg/ml [resistant]) and EUCAST 2014 (≤0.06 μg/ml [susceptible], >0.06 μg/ml [resistant]) differ. Both criteria were compared in this study to define which was preferable for addressing the choice of antibiotic to treat *S.* typhi infections. The isolates were also tested for the MIC of ciprofloxacin using the gradient E-strip test method (bioMerieux SA, Marcy-l'Etoile, France) based on the manufacturer's instructions. *Pseudomonas aeruginosa* ATCC 27853 was used as the quality-control strain for the determination of MIC with the expected range of 0.25–1 μg/ml. The interpretative criterion provided by CLSI 2014 for susceptible, intermediate and resistant strains was ≤0.06, 0.12–0.5 and ≥1 μg/ml, respectively.

2.3 Plasmid-Mediated Quinolone Resistance: Polymerase Chain Reaction (PCR)

Using multiplex polymerase chain reaction (PCR), all 33 typhoidal *Salmonella* isolates were screened for the presence of plasmid-mediated quinolone resistance (PMQR) genes *qnr*A, *qnr*B and *qnr*S, using primers as previously described [11]. Briefly, amplification of PMQR genes was performed using a Veriti Thermal Cyler (Applied Biosystems, Foster City, CA, USA) with the following conditions: initial denaturation step at 94 °C for 5 min, followed by 32 cycles of 94 °C for 45 s, 53 °C for 45 s and 72 °C for 60 s, and a final extension step of 72 °C for 7 min.

2.4 Quinolone-Resistance-Determining Regions (QRDR): PCR and Sequencing

Quinolone-resistance-determining regions (QRDR) of the DNA gyrase subunit II genes *gyr*A, *gyr*B and *par*C of the

DNA topoisomerase subunit IV were amplified using the primers described previously [12]. QRDR genes were amplified with the following conditions: initial denaturation step at 92 °C for 5 min, followed by 30 cycles of 92 °C for 1 min, 62 °C for 1 min and 74 °C for 2 min, and a final extension step of 74 °C for 1 min. The amplicons were subjected to direct DNA sequencing (ABI Prism 3100 Genetic Analyzer; Applied Biosystems) to detect mutations in the *gyr*A, *gyr*B and *par*C genes of both strands. Mutations in the amino acid level were analyzed by aligning the translated sequence with the *S.* typhi strain with the following accession numbers: *gyr*A- CAD07504, *gyr*B-NC_003198 and *par*C-NC_003198.1.

2.5 Phenotypic Efflux Pump Assay

Ciprofloxacin-resistant isolates with an MIC value >32 μg/ml were tested for efflux pump assay. This was carried out in the presence and absence of the efflux pump inhibitor (EPI) phenyl arginine β naphthylamide (PaβN) at a concentration of 20 μg/ml [7].

2.6 Statistical Analysis

All assays in this study were performed in duplicate. Significant differences between groups were determined at $p < 0.05$. To evaluate relationships between experimental parameters, results were analyzed for correlation and tested for significance using the Student's *t* test ($p < 0.05$). SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical evaluations.

3 Results

3.1 Antimicrobial Susceptibility Testing

Of the total 113 blood typhoidal *Salmonella* isolates, 98.2 % ($n = 111$) were susceptible to chloramphenicol and trimethoprim-sulfamethoxazole, 97.3 % ($n = 110$) to ampicillin, 100 % ($n = 113$) to ceftriaxone, and 13.3 % ($n = 15$) to nalidixic acid and ciprofloxacin. Of the 33 selected isolates, a wide ciprofloxacin MIC range was observed (Table 1). Overall, 25 of 27 *S.* typhi isolates were resistant to pefloxacin using the disc diffusion method.

3.2 PCR and Sequencing

Of 33 selected isolates screened for PMQR genes, only one isolate was found to have the *qnr*B gene. All except one non-susceptible isolates were found to have point mutations in either *gyr*A and/or *par*C genes. The two susceptible isolates did not harbor any mutations in *gyr*A, *gyr*B or *par*C. Both double and single amino acid substitutions were observed at positions 83 and 87 in all the isolates for *gyr*A. No mutations were detected for *gyr*B, while *par*C mutations were detected at positions 80 and 84.

3.3 Phenotypic Efflux Pump Assay

Efflux pump overexpression analysis was performed for eight isolates with ciprofloxacin MIC >32 μg/ml. A threefold decrease was the significant criterion for efflux pump activity. Only one isolate showed efflux pump activity, in which a notable fivefold decrease was found (8 to 0.25 μg/ml). The remaining seven isolates showed only a one- or twofold reduction, which was not considered significant for efflux pump activity.

4 Discussion

CLSI and EUCAST suggest different breakpoints, and the assignment of a pathogen to a defined category (susceptible, intermediate and resistant) depends on the specific guideline adopted. This could have an important impact on the selection of the drug to be used by the physician [13]. The choice of antibiotic for empirical therapy is based on patient treatment outcome; however, definitive therapy is based on the susceptibility pattern of the clinical isolate from the patient. This study will help in choosing the antibiotic for definitive therapy based on the susceptibility pattern of the isolate. In addition, this study discusses the use of appropriate breakpoints to determine the right susceptibility for ciprofloxacin, which ultimately helps in antibiotic selection for definitive treatment.

The differences between CLSI and EUCAST are primarily due to the various pharmacokinetic/pharmacodynamic approaches used to define these breakpoints. CLSI breakpoints are based on MIC distributions, pharmacokinetic/pharmacodynamic parameters and mechanisms of antimicrobial resistance, while EUCAST uses pharmacokinetic/pharmacodynamic simulations as a chief component of its breakpoint-setting process [13]. Both approaches use the Monte Carlo simulation, with a probability of target attainment (PTA) of free drug concentration above the MIC at least 40 % of the time (approximately 40 % $fT > MIC$). However, CLSI uses PTA mean values with a 90 % confidence interval (CI), whereas EUCAST uses 95 % and 99 % CIs [14].

Since pharmacokinetics/pharmacodynamics and limited clinical data do not support an intermediate category, EUCAST has removed the intermediate zone [15]. Consequently, antimicrobial susceptibility testing (AST) reports are simplified in EUCAST by reporting an isolate

Table 1 Antimicrobial susceptibility testing profile of typhoidal *Salmonella* isolates, molecular characterization, and CLSI and EUCAST interpretative criteria

Strain ID	Received date	Age/sex	Antimicrobial susceptibility testing by disc diffusion AMP–CHL–SXT–CTR–NAL–CIP–PEF	CIP MIC	Mutational analysis of QRDR genes			PMQR genes	Ciprofloxacin MIC interpretation	
					*gyr*A	*gyr*B	*par*C	*qnr*A, *qnr*B, *qnr*S	CLSI 2014	EUCAST 2014
Salmonella typhi										
ST1[a]	01-04-2014	49/M	NAL–CIP–PEF	>32	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–	Resistant	Resistant
ST2	02-07-2014	39/F	AMP–NAL–CIP–PEF	>32	Ser(83)-Phe	–	Glu(84)Lys	*qnr*B		
ST3	13-02-2014	1/M	AMP–NAL–CIP–PEF	>32	Ser(83)-Phe	–	Glu(84)Lys	–		
ST4	17-04-2014	22/M	NAL–CIP–PEF	>32	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–		
ST5	23-04-2014	28/F	NAL–CIP–PEF	>32	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–		
ST6			NAL–CIP–PEF	>32	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–		
ST7	09-09-2014	23/M	NAL–CIP–PEF	32	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–		
ST8	27-02-2014	24/F	NAL–CIP–PEF	12	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–		
ST9	03-10-2014	19/M	SXT–NAL–CIP–PEF	6	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–		
ST10	18-07-2014	23/M	NAL–PEF	6	Ser(83)-Phe	–	–	–		
ST11	29-07-2014	22/M	NAL–PEF	6	Ser(83)-Phe	–	–	–		
ST12	16-05-2014	30/M	NAL–PEF	1.5	Ser(83)-Tyr	–	–	–		
ST13	01-07-2014	46/M	NAL–PEF	1.5	Ser(83)-Phe	–	–	–		
ST14	29-07-2014	24/M	NAL–PEF	1.5	Asp(87)-Tyr	–	–	–		
ST15	21-07-2014	10/F	NAL–PEF	0.75	Ser(83)-Tyr	–	–	–	Intermediate	
ST16	04-11-2014	20/F	NAL–PEF	0.75	Ser(83)-Tyr	–	–	–		
ST17	09-11-2014	3/M	NAL–PEF	0.38	Ser(83)-Phe	–	Glu(84)-Gly	–		
ST18	19-12-2014	24/M	SXT–NAL–CIP–PEF	0.5	Ser(83)-Phe Asp(87)-Asn	–	Ser(80)-Ile	–		
ST19	29-07-2014	3/M	NAL–PEF	0.5	Asp(87)-Tyr	–	–	–		
ST20	11-08-2014	18/M	NAL–PEF	0.38	Ser(83)-Phe	–	–	–		
ST21	16-08-2014	30/F	NAL–PEF	0.38	Ser(83)-Tyr	–	Gly(72)-Ser	–		
ST22	11-10-2014	10/M	NAL–PEF	0.38	Ser(83)-Phe	–	Glu(84)-Gly	–		
ST23	02-10-2014	21/F	NAL–PEF	0.25	Ser(83)-Phe	–	–	–		
ST24	27-08-2014	26/M	NAL–PEF	0.25	Ser(83)-Tyr	–	Gly(72)-Ser	–		
ST25	01-10-2014	31/M	PEF	0.19	–	–	–	–		
ST26	12-07-2014	15/F	–	0.064	–	–	–	–	Susceptible	
ST27	30-05-2014	29/M	–	0.023	–	–	–	–		

Table 1 continued

Strain ID	Received date	Age/sex	Antimicrobial susceptibility testing by disc diffusion AMP–CHL–SXT–CTR–NAL–CIP–PEF	CIP MIC	Mutational analysis of QRDR genes			PMQR genes	Ciprofloxacin MIC interpretation	
					*gyr*A	*gyr*B	*par*C	*qnr*A, *qnr*B, *qnr*S	CLSI 2014	EUCAST 2014
Salmonella paratyphi A[b]										
SPA1	21-01-2014	39/M	NAL	0.75	Ser(83)-Phe	–	–	–	Intermediate	Resistant
SPA2	23-01-2014	39/M	NAL	0.75	Ser(83)-Phe	–	–	–		
SPA3	07-04-2014	13/M	NAL	0.75	Ser(83)-Phe	–	–	–		
SPA4	19-04-2014	34/M	NAL	0.75	Ser(83)-Phe	–	–	–		
SPA5	09-06-2014	18/M	NAL	0.75	Ser(83)-Phe Asp(87)Asn	–	Ser(80)-Ile	–		
SPA6	11-06-2014	16/M	NAL	0.75	Ser(83)-Phe	–	–	–		

ST Salmonella typhi, *SPA Salmonella* paratyphi A, *AMP* ampicillin, *CHL* chloramphenicol, *SXT* trimethoprim-sulfamethoxazole, *NAL* nalidixic acid, *CIP* ciprofloxacin, *PEF* pefloxacin, *CTR* ceftriaxone, *QRDR* quinolone-resistance-determining region, *PMQR* plasmid-mediated quinolone resistance, *M* male, *F* female, *MIC* minimum inhibitory concentration, *CLSI* Clinical and Laboratory Standards Institute, *EUCAST* European Committee on Antimicrobial Susceptibility Testing

[a] Strain with efflux pump activity—fivefold decrease in CIP MIC (8–0.25 μg/ml) in the presence of efflux pump inhibitor

[b] Pefloxacin testing not undertaken for *S.* paratyphi isolates

as either susceptible or resistant. This strategy will change AST reports, mostly by reporting isolates as resistant that were formerly considered intermediate [15]. The adoption of CLSI or EUCAST interpretative criteria may therefore lead to different results and conclusions [13].

The present study compared the CLSI and EUCAST criteria of ciprofloxacin susceptibility for typhoidal *Salmonella* with their molecular resistance mechanism. Most of the study isolates might belong to a single clone, ST1 (93 %), followed by ST2 (6 %), as observed from a previous study at the Christian Medical College, Vellore (unpublished data).

In this study, 65.4 and 23.5 % of typhoidal *Salmonella* cases were found to be intermediate and resistant to ciprofloxacin, as per MIC testing and according to CLSI 2014. However, with EUCAST 2014-defined breakpoints, the resistance rate was higher (93.9 %).

Interestingly, we found that 96 % of isolates in the intermediate category that were assigned as per CLSI breakpoints had mutations in *gyr*A and *par*C, which might translate to possible/probable clinical failure in patients if treated with ciprofloxacin. Renuka et al. [16] reported a similar observation of mutations in ciprofloxacin-reduced susceptible isolates; however, Menezes et al. [7] reported a single mutation in the *gyr*A gene of an intermediate *S.* typhi. Recently, García-Fernández et al. [3] reported similar mutations in ciprofloxacin-intermediate isolates as per CLSI. Following the criteria suggested by EUCAST, $\leq$0.06 being susceptible and >0.06 being resistant, may result in more appropriate therapy with reduced morbidity.

In this study, most ciprofloxacin resistance was mediated by chromosomal mutations. Of the mutations observed among *S.* typhi in *gyr*A, seven of eight Asp(87)-Asn (GAC-AAC) substitutions were present in isolates with a ciprofloxacin MIC >6 μg/ml (>32 μg/ml = 5, 12 μg/ml = 1, 6 μg/ml = 1), with the exception of ST18. This shows a strong association of Asp(87)-Asn mutation in the *gyr*A gene, with a high level of resistance to ciprofloxacin. In comparison, Ser(83)-Phe (TCC-TTC) substitution in the *gyr*A gene was seen in 17 isolates, of which 12 were seen in isolates with a MIC >1.5 μg/ml, while five were seen in isolates with an intermediate MIC. This is the commonly observed mutation among *S.* typhi with both low- and high-level ciprofloxacin resistance [16, 17]. In contrast, Ser(83)-Tyr (TCC-TAC) mutation in the *gyr*A gene was majorly observed in isolates with an intermediate MIC (0.75 μg/ml = 2, 0.38 μg/ml = 1, 0.25 μg/ml = 1). In addition, Asp(87)-Tyr (GAC-TAC) mutation (sequences submitted to NCBI–KT162084, KT162085), less common in *S.* typhi in *gyr*A, was observed in two of the isolates (ST14 and ST19).

In the *par*C gene, Ser(80)-Ile (AGC-ATC) and Glu(84)-Lys (GAA-AAA) mutations were mostly associated with

an MIC >6 μg/ml, and Glu(84)-Gly (GAA-GGA) and Gly(72)-Ser (GGT-AGT) were seen only in isolates with an intermediate MIC. All the *S.* paratyphi isolates ($n = 6$) exhibited a MIC of 0.75 μg/ml, which did not fall into any category of CLSI 2014. Mutations Ser(83)-Phe ($n = 6$) and Asp(87)-Asn ($n = 1$) in the *gyr*A gene, and Ser(80)-Ile ($n = 1$) in the *par*C gene, were commonly observed among *S.* paratyphi.

To the best of our knowledge, the *par*C mutation Glu(84)-Gly (sequences submitted to NCBI–KT162086, KT162087) was observed for the first time in *S.* typhi, in India, marked with a low MIC of 0.38 μg/ml in this study. The consequence or phenotypic expression of this mutation is not known, and further molecular docking studies are required in order to identify the effect of mutation and ciprofloxacin binding strength.

One isolate, ST2 (with an MIC >32 μg/ml), was found to harbor a PMQR gene (*qnr*B), along with a single mutation each in *gyr*A [Ser(83)-Phe] and *par*C [Glu(84)-Lys]. From these results, high-level MIC was concordant ($p < 0.05$) with a multiple number of mutations, which might be due to the cumulative effect. As expected, the susceptible isolates did not show any mutations.

A maximum number of mutations (three) were observed in ST1, ST4, ST5, ST6, ST7, ST8 and ST9, with high MIC values (>6 μg/ml). Two isolates (ST18 and SPA5) with 0.5 and 0.75 μg/ml MICs also had three mutations. It is noted that the high number of mutations does not always associate with high MIC values. It was previously reported that *S.* typhi isolates with a MIC as low as 0.5 μg/ml can harbor three mutations [18]; however, as per EUCAST criteria, ST18 and SPA5 were phenotypically resistant to ciprofloxacin due to the presence of the mutations.

In contrast, isolates ST10 and ST11 had a MIC of 6 μg/ml when only a single mutation was present in *gyr*A. A single mutation would result in decreased susceptibility and low MIC. In a similar study, it was reported that three mutations were required to confer resistance to ciprofloxacin in *S.* typhi [19]; however, in this study we were unsure whether any other antimicrobial resistance mechanism were acting in ST10 and ST11. The contribution of a high MIC value of 6 μg/ml might be associated with another unreported mechanism of resistance to ciprofloxacin.

Resistance to ciprofloxacin might also be due to intrinsic resistance, which involves overexpression of the efflux pump. The most commonly described efflux pump in *Salmonella*, is the Acr-AB TilC pump, which belongs to the Resistance-Nodulation-Cell Division (RND) super family [20]. Data on the genotypic characterization of efflux pumps in *Salmonella* is limited in India; also, very little evidence has previously been reported on phenotypic characterization [7]. The efflux pump was reported to contribute only a high-level of ciprofloxacin resistance in *S.* typhi [21]. In this study, there was no significant change in efflux pump activity for ciprofloxacin in tested isolates, with the exception of one, which showed a fivefold decrease.

In 2014, EUCAST suggested pefloxacin as a dependable surrogate marker for fluoroquinolone susceptibility, rather than ciprofloxacin, for identifying chromosomal as well as plasmid-mediated resistance [22], whereas nalidixic acid (30 μg) could only identify chromosomal but not plasmid-mediated resistance [23]. In this study, we found that pefloxacin was significantly ($p < 0.05$) more reliable than ciprofloxacin as 40 % ($n = 11$) of the selected *S.* typhi isolates that were categorised as 'intermediate', according to the ciprofloxacin MIC (CLSI), were all found to be resistant using pefloxacin disc diffusion (CLSI and EUCAST), which correlates with the ciprofloxacin MIC interpretation as per EUCAST. Furthermore, pefloxacin can be used to extrapolate susceptibility results for nalidixic acid and ciprofloxacin disc diffusion tests and levofloxacin, ofloxacin and ciprofloxacin MIC test. Currently, the United States Committee on Antimicrobial Susceptibility Testing (USCAST) have suggested fluoroquinolone susceptibility breakpoints for Enterobacteriaceae, non-fermenting gram-negatives and gram-positives. It will be interesting to know what USCAST will suggest for *Salmonella spp*.

Pefloxacin does not have an intermediate category and it clearly defines the isolate as susceptible or resistant, whereas the CLSI criteria can be misleading with the selection of fluoroquinolone for treatment. In addition, there is no susceptible dose-dependent category for ciprofloxacin to *Salmonella spp.* as per the CLSI criteria. For the isolates with an intermediate MIC, treatment with a high-dose of ciprofloxacin may or may not be successful.

Moreover, the molecular mechanism of resistance to ciprofloxacin correlates better with the EUCAST criteria than the CLSI criteria, which also complies with the pefloxacin disc diffusion results (CLSI and EUCAST).

4.1 Limitations

Only a limited number of isolates were tested for pefloxacin, thus further evaluation is required with a greater number of isolates. The interpretative criteria defined for pefloxacin are narrow (1 mm difference between 'susceptible' and 'resistant'), necessitating stringent quality control [24].

5 Conclusions

Currently, the vast majority of *S.* typhi clinical isolates fall into the intermediate susceptible category, using the CLSI criteria. On molecular characterization, almost all these

isolates were found to harbor gene encoding resistance, implying clinical failure if treated with fluoroquinolones. It is clearly evident from this study that the EUCAST breakpoint criteria should be followed, rather than CLSI, to make the appropriate therapeutic choice. In our experience, pefloxacin is promising as a surrogate marker for fluoroquinolone susceptibility (as per both EUCAST and CLSI criteria).

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Compliance with Ethical Standards

Conflict of interest Balaji Veeraraghavan, Shalini Anandan, Dhiviya Prabaa Muthuirulandi Sethuvel, Nivetha Puratchiveeran, Kamini Walia and Naveen Kumar Devanga Ragupathi declare that there are no conflict of interest.

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