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Pefloxacin as a Surrogate Marker for Ciprofloxacin Resistance in Salmonella: Study from North India

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***Abstract*—**Fluoroquinolones form the mainstay of therapy for the treatment of infections due to *Salmonella enterica* subsp. *enterica*. There is a complex interplay between several resistance mechanisms for quinolones and various fluoroquinolones discs, giving varying results, making detection and interpretation of fluoroquinolone resistance difficult. For detection of fluoroquinolone resistance in *Salmonella* ssp., we compared the use of pefloxacin and nalidixic acid discs as surrogate marker. Using MIC for ciprofloxacin as the gold standard, 43.5% of strains showed MIC as ≥1 µg/ml and were thus resistant to fluoroquinoloes. Based on the performance of nalidixic acid and pefloxacin discs as surrogate marker for ciprofloxacin resistance, both the discs could correctly detect all the resistant phenotypes; however, use of nalidixic acid disc showed false resistance in the majority of the sensitive phenotypes. We have also tested newer antimicrobial agents like cefixime, imipenem, tigecycline and azithromycin against *Salmonella* spp. Moreover, there was a comeback of susceptibility to older antimicrobials like ampicillin, chloramphenicol, and cotrimoxazole. We can also use cefixime, imipenem, tigecycline and azithromycin in the treatment of multidrug resistant *S. typhi* due to their high susceptibility.

***Keywords*—**Pefloxacin, salmonella, surrogate marker.

I. Introduction

SALMONELLA enterica subsp. *enterica* represents a major disease burden in India. The treatment with appropriate antimicrobials, at the earliest, is crucial for complete recovery from enteric fever, to prevent complications and drug resistance. Fluoroquinolones are highly efficient and are the drugs of choice [1]. However, increasing drug resistance and difficulty in laboratory detection of various resistance phenotypes has compounded this problem [2].

Currently, three main mechanisms of resistance to fluoroquinolones in salmonella isolates are recognized; mutations that alter target site, reduced drug penetration due to mutations and plasmid-mediated resistance that protect the bacterial isolates from lethal effect of fluoroquinolones [3]. The essential bacterial enzymes DNA gyrase and DNA topoisomerases IV is the target of quinolone action. These two enzymes are required for replication, transcription, recombination and repair of DNA [4]. The two subunits of DNA gyrase are: Gyr A which is encoded by gyr A gene and Gyr B encoded by gry B gene [5]. The corresponding subunits of topoisomerases IV are: Par C and Par E, encoded by par C and par E gene, respectively.

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Resistance involving the mutation in a region of gyr A and par C subunits is present in quinolone resistance determining regions (QRDRs). The second mechanism decreases the accumulation of fluoroquinolone in the bacterial cell due to increased efflux, caused by the mutation leading to overexpression of AcrAB-TolC efflux pump [6]. Lastly, in 1990s, plasmid-mediated quinolone resistance emerged and this was due to the presence of various genes like qnr, qepA and aacs(6′)-Ib-cr [7]. These isolates having plasmid-mediated resistance showed reduced susceptibility to ciprofloxacin (MIC 0.125-1.0 mg/L), but only a modest or no increase in MIC to nalidixic acid (MIC 8-32mg/L). These patients had more treatment failures and longer time for fever clearance [1].

The interplay between several of these resistance mechanisms produces different genotypes having variation in their MICs [8]. A single mutation in gyr A, even with the functional efflux system has increased the resistance (MIC of ciprofloxacin in the range of 0.12-0.5mg/L) moderately. After the second mutation in gyr A and par C, there will be a high level of resistance (MIC ≥ 4 mg/L). Therefore, the high level of fluoroquinolone resistance arose due to the cumulative effect of multiple mutations in QRDR which has decreased permeability of membrane and active efflux and/ or plasmid encoded genes [9], [10]. To add to these complexities, *in vitro* testing of salmonella isolates in the laboratory, having varying resistant determinants, with the use of different fluoroquinolones discs have given confusing results. There is an overlay in zone diameter with 5 µg ciprofloxacin disc between wild-type isolates and isolates with low-level resistance. Use of nalidixic acid as a surrogate marker has also proved to be unreliable because it does not adequately detect plasmid-mediated resistance and gives susceptible results in these particular isolates [11], [12].

In developing countries, like India, detection of salmonella strains with low-level resistance is important and challenging as MIC detection and/ or detection of resistant genes cannot be routinely done. In 2015, CLSI recommended the use of 5 µg pefloxacin disc as a surrogate marker for ciprofloxacin resistance [13]. Therefore, the objectives of our study were to compare pefloxacin as a surrogate marker viz-a-viz nalidixic acid for testing ciprofloxacin resistance using MIC as the gold standard. This is the first study of its kind from India. Additionally, susceptibility testing of salmonella species to newer drugs like tigecycline, azithromycin, cefixime and imipenem has also been done.

II. Material and Methods

The present study was conducted in a tertiary care hospital (Government Medical College Hospital, Chandigarh) catering to the population of North India. A total of 48 isolates of *Salmonella typhi* and *Salmonella paratyphi* A isolated from the blood culture from January 2014 to June 2015 were included in the study.

The blood sample was collected from suspected adult and pediatric patients who were having signs and symptoms suggestive of typhoid fever. All the patients who were attending the outpatient departments or who were admitted to Government Medical College Hospital, Chandigarh were included in the study. The blood sample was taken prior to the administration of antimicrobial drugs. Blood samples were inoculated into brain heart infusion broth and incubated at 37 °C. Three subcultures were made onto Blood agar and Mac Conkey agar (Hi-Media, Mumbai, India), firstly after overnight incubation, then after 48 hours and lastly after 7 days [14]. The isolates obtained were identified by conventional biochemical tests and confirmed by agglutination with Salmonella antisera (Denka Seiken Co, Ltd., Japan) [15].

A. Kirby Bauer Disc Diffusion Test

Antibiotics susceptibility testing was done according to the latest CLSI guidelines [13] for the following antibiotics: ampicillin (10 µg), cotrimoxazole (1.25/23.75 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), pefloxacin (5 µg), ceftriaxone (30 µg), cefixime (10 µg), azithromycin (15 µg), imipenem (10 µg). The disc of tigecycline (15 µg) was also used and interpreted by EUCAST guidelines [16].

B. MIC Test

MIC testing of all 48 isolates to ciprofloxacin was done by E-test method (AB Biodisk, Solna, Sweden). MIC testing by E-test was also done for imipenem (interpreted by CLSI 2015) and for tigecycline (interpreted EUCAST guidelines) [5], [6].

III. Results

A total of 48 isolates were included in the study; 45 strains of *Salmonella typhi* and 3 strains of *Salmonella paratyphi* A. The OPD: IPD ratio was 2:1 and male: female ratio was also 2:1. Most of the patients were in the pediatrics age group (65%).

There was 100% susceptibility to older antimicrobials like cotrimoxazole, chloramphenicol and newer antimicrobials like cefixime, ceftriaxone, and azithromycin by disc diffusion. Whereas, the susceptibility of salmonella isolates to ampicillin was 70%. For tigecycline and imipenem the MIC was in the susceptible range (≤1 µg/ml).

Susceptibility to fluoroquinolones i.e. ciprofloxacin, nalidixic acid and pefloxacin showed varying results. Ciprofloxacin disc showed maximum susceptibility (62.5%), pefloxacin disc showed susceptibility to 56.2% of isolates and least susceptibility was seen with a nalidixic acid disc (2.1%) for salmonella isolates **(**Table I).

A total of 21 strains were resistant using Ciprofloxacin MIC as the gold standard (E-test). All these 21 strains were resistant to nalidixic acid and pefloxacin using CLSI 2015 disc diffusion criteria. But, only 4 amongst these strains showed resistance to ciprofloxacin disc and rest 17 isolates were sensitive to ciprofloxacin by CLSI disc diffusion criteria.

Additionally, 26 strains of *Salmonella* spp. were sensitive to ciprofloxacin by MIC method and all of these were also sensitive to pefloxacin by disc diffusion. But all of these showed resistance to nalidixic acid. Amongst these 26 strains, 14 were resistant and 12 were sensitive to ciprofloxacin. One strain of *Salmonella* spp. was found sensitive by MIC method and also showed susceptibility to all three discs i.e. nalidixic acid, pefloxacin, and ciprofloxacin (Table II).

TABLE I
Susceptibility of Salmonella Strains to Various Antimicrobial

Antimicrobial	*Salmonella typhi* (n=45)	*Salmonella paratyphi* A (n=3)	Total susceptibility 48 (%)
Ampicillin	32	2	34(70%)
Cotrimoxazole	45	3	48(100%)
Chloramphenicol	45	3	48(100%)
Ciprofloxacin	30	0	30 (62.5%)
Nalidixic acid	1	0	1(2.1%)
Pefloxacin	24	3	27(56.2%)
Cotrimoxazole	45	3	48(100%)
Cefixime	45	3	48(100%)
Azithromycin	45	3	48(100%)
Imipenem	45	3	48(100%)
Tigecycline	45	3	48(100%)

IV. Discussion

In 1962, the first report of chloramphenicol resistant *S. typhi* from India was documented. Thereafter, strains resistant to chloramphenicol, cotrimoxazole and ampicillin (MDR) spread throughout India in the late 1980s and 1990. Hence, ciprofloxacin became a drug on choice in the 1990s. This magic bullet became ineffective rapidly due to therapeutic failure. Many strains showing MIC in the range of 0.12-1.0µg/ml (Decreased Ciprofloxacin Susceptibility, DCS) showed zone diameter in the sensitive range by ciprofloxacin disc susceptibility and resistance to nalidixic acid (Nalidixic acid resistant, NAR) strains [2].

TABLE II
Distribution of Salmonella Strains Showing Susceptibility to Different Floroquinolones Disc and Their MIC

No. of isolates	Nalidixic acid	Pefloxacin	Ciprofloxacin	Ciprofloxacin MIC
21	Resistant	Resistant	17- sensitive 4- resistant	Resistant
26	Resistant	Sensitive	14-resistant 12- sensitive	Sensitive
1	Sensitive	sensitive	Sensitive	sensitive

Our study has documented that amongst the older antibiotics, 70% of Salmonella isolates was sensitive to ampicillin. All the isolates were sensitive to cotrimoxazole and chloramphenicol. No MDR strain (strain resistant to

cotrimoxazole, ampicillin, and chloramphenicol) was isolated in our study. In a study by Choudhary et al. from South India, high sensitivity to older antibiotics was reported i.e. 90% to ampicillin, 95% to cotrimoxazole and 100% to chloramphenicol [17]. Bhattacharya et al. from Southern India reported that 11.96% of *Salmonella typhi* and 15.62% of *Salmonella paratyphi* A as MDR [18]. Dutta et al. postulated that the reason for the reemergence of susceptibility to older drugs could be due to the emergence of de-novo susceptible strains or loss of high molecular plasmids encoding cotrimoxazole, ampicillin, and chloramphenicol resistance [19]. Similarly, newer drugs like cefixime, ceftriaxone, tigecycline and imipenem showed 100% susceptibility. Bhattacharya et al. documented sensitivity for third generation cephalosporin as between 94 to 97% [18]. In a similar study from North India, Capoor et al. reported MIC of all salmonella strains in the susceptible range for tigecycline and carbapenem [20]. Tigecycline is a glycycline derivative of minocycline. Tigecycline is a good treatment option for salmonella infection because of many reasons. Firstly, it circumvents the acquired efflux and target mediated resistance, which is the most frequent mechanism of drug resistance in tetracycline. Additionally, it is also unaffected by the presence of co-resistance to unrelated antimicrobial like ß-lactam, aminoglycosides, and quinolone. Moreover, since tigecycline has *in vitro* coverage to most ESBL producing Gram-negative bacteria, use of this antimicrobial could reduce the intensity of selection for ESBL – producing organisms [21].

For the first time in 2015, azithromycin susceptibility by disc diffusion was included by CLSI [13]. The role of azithromycin in the treatment of uncomplicated enteric fever in India is not clear. On one hand, the laboratory interpretation of report regarding the *in vitro* susceptibility of salmonella to azithromycin is difficult due to non-availability of breakpoint concentrations. Additionally, the MIC90 values for azithromycin against Salmonella isolates from India is high (24 µg/ml) as compared to the western countries (4-8µg/ml) [22]-[24]. Various studies by different authors to see the sensitivity of salmonella species to azithromycin showed varying degree of resistance. Choudhary et al. reported 52% of salmonella is resistant to azithromycin [17].

The use of azithromycin in the treatment of enteric fever is favored by clinicians because of its high intracellular tissue penetration and long elimination half-life (72 hours). In a study by Chinh et al., 5-day course of azithromycin showed superior result as compared to ofloxacin in the treatment of typhoid fever due to MDR and nalidixic acid resistant *S. typhi* [25]. Shah et al. concluded from the systematic review from the cochrane library that azithromycin reduces the clinical failure rate and duration of hospital stay in comparison to fluoroquinolones and relapse rate in comparison to ceftriaxone when used in the treatment of typhoid fever in populations with MDR *Salmonella typhi* [26]. Our study demonstrated that for the first time using CLSI 2015 criteria, all strains were found to be susceptible to azithromycin.

Using MIC of ciprofloxacin as the gold standard, 43.75% (21/ 48) of salmonella strains were in the resistant range (MIC >1 µg/ml). Bhattacharya et al. reported <2% of their Salmonella isolates as being resistant to ciprofloxacin [18]. In our study, no isolate with decreased ciprofloxacin susceptibility (DCS) along with nalidixic acid disc resistance was found.

We tested three fluoroquinolone discs – nalidixic acid, pefloxacin and ciprofloxacin using CLSI 2015 zone diameter cut-offs to compare which of these three is the best surrogate marker of ciprofloxacin resistance. Nalidixic acid disc could detect 21/21 resistant strains correctly but showed 26/27 sensitive strains to be falsely resistant though they were susceptible based on ciprofloxacin MIC values. Whereas, ciprofloxacin disc could detect only 4/21 truly resistant strains (19.04%) and out of the rest 27 sensitive strains it showed 14 strains to be falsely resistant. Pefloxacin disc could detect all 21/21 (100%) of these resistant strains correctly and showed all sensitive strains (27/27) to be susceptible. (Table II).

Based on the performance of nalidixic acid and pefloxacin discs as surrogate marker for ciprofloxacin resistance, both the discs could correctly detect all the resistant phenotypes; however, use of nalidixic acid disc showed false resistance in the majority of the sensitive phenotypes. This could direct the clinician to a wrong choice of antibiotic and also epidemiologically show a higher rate of ciprofloxacin resistance than the actual incidence.

Skov et al. recommended the use of pefloxacin disc as an excellent surrogate marker for fluoroquinolone resistance for disk diffusion assays and this was further implemented for use in EUCAST (2014) and CLSI (2015) guidelines. CLSI (2015) guidelines have documented that no single screening test detects all possible fluoroquinolone resistance mechanisms. But in a resource-constrained setting, pefloxacin is a suitable option for the detection of fluoroquinolone resistance in *S. typhi* [12].

The limitation for the use of pefloxacin disc as a surrogate marker is that for the resistance mediated by the aac(6')-Ib-cr gene use of only ciprofloxacin and norfloxacin is recommended because these are the fluoroquinolones which possess the piperazynil amide side chain which is the target for the enzyme encoded by aac(6′)Ib-cr.1 But this type of resistance is very rare.

In conclusion, there is a reemergence of susceptibility to first-line antibiotics (ampicillin, chloramphenicol and cotrimoxazole) for *S. typhi*. Other antimicrobial agents like third generation cephalosporins, azithromycin and tigecycline appear to be effective therapeutic options. Further studies are required to be done to establish and corroborate the superiority of pefloxacin disc over nalidixic acid disc as a surrogate marker for ciprofloxacin resistance.

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