Quinolone Susceptibility in *Salmonella* Isolates Based on Minimum Inhibitory Concentration Determination

Varsha Gupta[1] Kritika Pal[1] Alisha Bhagat[1] Anku Goel[1] Jagdish Chander[1]

[1]Department of Microbiology, Government Medical College and Hospital, Chandigarh, India

Address for correspondence Kritika Pal, MBBS, MD, Department of Microbiology, Government Medical College and Hospital, Chandigarh 160030, India (e-mail: drkritika4390@gmail.com).

J Lab Physicians:2020;12:263–267

Abstract

Introduction Typhoid fever, caused by *Salmonella typhi* and *paratyphi*, is a generalized infection with case fatality of about 10%. The symptoms may be severe, with life threatening sequelae of infection in a proportion of cases. Antimicrobial agents are the mainstay of therapy in enteric fever so as to prevent the complications associated with severe illness and mortality in the patients. Fluoroquinolones (e.g., ciprofloxacin) are very effective against completely susceptible *Salmonella* bacteria. However, their efficacy is doubtful once any resistance is detected. Pefloxacin testing has ultimately helped in the accurate identification of quinolone susceptibility for a better therapeutic success rate. In the present study we have tried to evaluate the quinolone susceptibility in *Salmonella* isolates based on minimum inhibitory concentration (MIC) determination.

Materials and Methods The method used in the study is quinolone susceptibility in *Salmonella* isolates based on MIC determination. *Salmonella* isolates show intermediate susceptibility to ciprofloxacin using disk diffusion. Both ciprofloxacin and pefloxacin MIC evaluation has been done to corroborate the results with pefloxacin disk diffusion testing.

Results There was a positive correlation between the susceptibility to ciprofloxacin and pefloxacin. However, the isolates with intermediate susceptibility had variations in terms of susceptibility to pefloxacin. MIC values for pefloxacin and our findings suggested that pefloxacin susceptible on disk diffusion as per Clinical and Laboratory Standards Institute guidelines showed lower values for MIC using Pefloxacin HICOMB test and pefloxacin resistant isolates showed higher MIC values.

Keywords
- MIC determination
- pefloxacin
- pefloxacin susceptible
- quinolone susceptibility
- *Salmonella* isolates

Introduction

Typhoid fever, caused by *Salmonella typhi* and *paratyphi*, is a generalized infection with case fatality of approximately 10%.[1] The symptoms may be severe, with life threatening sequelae of infection in a proportion of cases. Antimicrobial agents are the mainstay of therapy in enteric fever so as to prevent the complications associated with severe illness and mortality in the patients. Unfortunately, particularly in developing countries, the reduced susceptibility of *Salmonella enterica* to commonly used antibiotics continues to be a major problem. Earlier multidrug resistant strains of *Salmonella enterica* (resistant to chloramphenicol, ampicillin, and cotrimoxazole) were increasingly being reported.[2] However, due to restriction of their use and negative selection pressure, these drugs have again shown susceptibility for the treatment of typhoid fever in endemic areas. Later on, fluoroquinolones were used as the drug of choice for having the high level of clinical efficacy against most of the enteric pathogens including *Salmonella*. Subsequently, during the last few years, nalidixic acid-resistant strains associated with decreased susceptibility to fluoroquinolones in the patients treated with quinolones have been increasingly reported.[1,3]

DOI https://doi.org/10.1055/s-0040-1721163
ISSN 0974-2727 .

Thieme Medical and Scientific Publishers Pvt. Ltd. A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

Fluoroquinolones (e.g., ciprofloxacin) are very effective against completely susceptible *Salmonella* bacteria. However, their efficacy is doubtful once any resistance is detected. Quinolones act on the deoxyribonucleic acid (DNA) gyrase and topoisomerase enzymes, leading to inhibition of replication and transcription activities thus causing DNA fragmentation. There are various mechanism of resistance, most commonly being the mutations in quinolone resistance determining region of target genes gyrA, gyrB, parC, and parE14, presence of plasmid-mediated qnr genes, qepA and aacs[4]-Ib-cr genes and overexpression of efflux pumps.[5]

Many studies have reported that bacteria having plasmid-mediated resistance show reduced susceptibility to ciprofloxacin (minimum inhibitory concentration or MIC of 0.125–1.0 µg/mL), thus making it difficult to be picked up by the nalidixic acid test. Due to this, the Clinical and Laboratory Standards Institute (CLSI) and EUCAST recommended a new screening surrogate marker of pefloxacin (5 µg) disk diffusion for detecting both chromosomal as well as plasmid-mediated resistance. This was confirmed by testing of pefloxacin, wherein 80% of the nalidixic acid-resistant strains and ciprofloxacin intermediate susceptible isolates were resistant to pefloxacin. Consequently, pefloxacin testing has ultimately helped in the accurate identification of quinolone susceptibility for a better therapeutic success rate.[2]

In developing countries like India, detection of low level fluoroquinolone resistance by manual determination of MIC and detection of resistant genes is cumbersome and time consuming so cannot be performed routinely. Recommendations on the use of pefloxacin (5 µg) for the detection of *Salmonella* resistance were made by CLSI in 2015. There are some difficulties faced while using pefloxacin as the zone of inhibition (ZOI) on disk diffusion test. ZOI < 23 mm is for resistant isolates and > 24 mm for susceptible isolates as per CLSI. This range is too narrow and sometimes in laboratory setting this could lead to subjective errors while reading the plates. Also, pefloxacin is readily not available in United States and is not able to detect resistance related to aacs[4]-Ib-cr genes. Therefore, it is mentioned in CLSI M100 that no one test can accurately determine all the various types of resistance to flouroquinolones.[6]

So, in the present study we have tried to evaluate the quinolone susceptibility in *Salmonella* isolates based on MIC determination. We have studied the *Salmonella* isolates showing intermediate susceptibility to ciprofloxacin using disk diffusion. Both ciprofloxacin and pefloxacin MIC evaluation has been done to corroborate the results with pefloxacin disk diffusion testing.

Material and Methods

A total of 56 strains of *Salmonella enterica* were included in the study during time period of December 2018 to December 2019. All the strains were isolated from blood cultures of patient suspected of having enteric fever. The isolates were stocked in glycerol stocks and refrigerated at 70 degrees.

Biochemical Identification

The strains were revived and tested using standard biochemical method. Out of 56, 46 strains were identified as *Salmonella enterica serovar typhi* and ten strains were identified as *Salmonella enterica serovar paratyphi A*. *Salmonella* was identified based on standard methods including colonial morphology, Gram's staining, biotyping, and serotyping (Denka Seiken Co. Ltd., Japan)[4]

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the isolated strains was performed using the disk diffusion method (modified Kirby–Bauer method) on Mueller–Hinton agar (HiMedia, India) as recommended by the CLSI, Wayne, United States.[4] Susceptibility of the fluoroquinolones including nalidixic acid (30 µg), pefloxacin (5 µg), ciprofloxacin (5 µg), azithromycin (15 µg), chloramphenicol (30 µg), cotrimoxazole(1.25 µg/23.75 µg), cefixime (5 µg), and ceftriaxone (30 µg) (HiMedia Laboratories, India) was done. The results of the antibiotic susceptibility were determined on the basis of interpretative zone diameters as suggested by CLSI. For standardization, *Escherichia coli* ATCC-25922 was used as the control organism for antibiotic sensitivity.

Further, MICs of all isolates was checked using broth microdilution method as per CLSI guidelines.[7] Ciprofloxacin concentrations ranged from 0.06 to 16 µg/mL. Pefloxacin HiComb (HiMedia),was used for determining the MIC of pefloxacin, which is available as Part A and Part B with concentration of 240 to 0.01 µg/mL and 30 to 0.001 µg/mL, respectively. Antimicrobial susceptibility testing was performed according to the manufacturer's instructions and interpreted using CLSI guidelines. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 served as quality control strains.

Results

On antimicrobial susceptibility testing by disk diffusion, all the isolates of *Salmonella typhi* were susceptible to azithromycin, chloramphenicol, cotrimoxazole, cefixime, and ceftriaxone. Similarly, all isolates of *Salmonella paratyphi A* were also susceptible to azithromycin, chloramphenicol, cotrimoxazole, tetracycline, cefixime, and ceftriaxone. Susceptibility to nalidixic acid was also tested and 34 of 46 and 8 of 10 patients were resistant.

Ciprofloxacin susceptibility was tested and interpreted as per CLSI guidelines based on disk diffusion methods. Of the 46 isolates, only eight were susceptible to ciprofloxacin, 22 isolates were intermediate (ZOI), and 16 were resistant. Out of these 22 intermediate isolates, based on ciprofloxacin MIC values, eight showed susceptibility, two were in intermediate susceptibility range, ten were resistant, and two were not differentiated clearly. Further amongst these, when using pefloxacin, eight isolates were susceptible and 14 were resistant using disk diffusion method (►**Table 1**).

Of the ten isolates of *S. paratyphi*, only six were susceptible to ciprofloxacin, four isolates were intermediate (ZOI), and none were resistant. Out of these four intermediate isolates, based on ciprofloxacin MIC values, two showed

susceptibility and two were resistant. Further amongst these, when using pefloxacin, two isolates were susceptible and two were resistant using disk diffusion method (►**Table 2**).

The breakpoints for susceptible zones of inhibition for pefloxacin and ciprofloxacin corresponded to each other regarding susceptibility and resistance. However, there was lot of discrepancy regarding intermediate ciprofloxacin range as depicted in ►**Table 3**. The susceptibility rates of the two drugs in the range of intermediate susceptibility were compared by Normal test of proportion and these rates were found to be statistically significant ($p < 0.05$). The resistance rate was found to be significantly higher for pefloxacin.

Twenty-two isolates that were intermediate to ciprofloxacin by disk diffusion method were subjected to MIC determination using broth dilution test. Eight had MIC < 0.06, two had MIC between 0.12 to 0.5 µg/mL, and ten isolates had MIC in the range of 1 to 16 µg/mL; however, range could not be determined for two isolates. Pefloxacin zone diameters and ciprofloxacin MIC results were also compared to evaluate the efficacy of pefloxacin as a surrogate marker for fluoroquinolones susceptibility. Eight isolates were found to be susceptible to pefloxacin whereas 14 isolates were found to be resistant to pefloxacin (►**Table 2**).

For *Salmonella paratyphi A*, out of four intermediate isolates, based on ciprofloxacin MIC values; two showed susceptibility, two were resistant and the results corroborated with pefloxacin disk diffusion test.

MIC of pefloxacin was determined using Pefloxacin HICOMB methods, which is a gradient diffusion susceptibility testing method. Isolates with pefloxacin ZOI > 24 mm showed MIC < 5 using Part A of HICOMB and < 1 of Part B. Resistant isolates (ZOI < 23) showed MIC ≥ 5 and MIC ≥ 1 of part A and part B, respectively by Pefloxacin HICOMB gradient diffusing susceptibility testing method (►**Table 3**).

For pefloxacin susceptible isolates, (ZOI > 24 mm), MIC of ciprofloxacin was less than 0.06 µg/mL and pefloxacin MIC was A ≤ 0.1, B ≤ 0.1 as shown in the ►**Table 3**. For the pefloxacin-resistant isolates (ZOI < 23 mm) MIC for ciprofloxacin was within the range of 0.5 to 16 µg/mL except in one case where it was 0. 125 µg/mL and MICs for pefloxacin were A > 5, B > 1 (►**Fig. 1**).

Discussion

Ciprofloxacin became the drug of choice for the treatment of *Salmonella* infection in 1990. However, there was therapeutic failure associated with strains showing MIC in the range 0.12 to1 µg/mL (decreased ciprofloxacin susceptibility [DCS]). For ciprofloxacin, the CLSI revised the breakpoints for designating the clinical isolates with MIC ≤0.06 µg/mL as susceptible in 2012. DCS is commonly seen in India and is associated with clinical failures. However, this could not be determined using nalidixic acid as a marker for resistance determination but can be determined by pefloxacin. Early on, this was missed as the dependence lied solely on nalidixic acid resistance using disk diffusion testing.[8-12]

Fluoroquinolones due to the properties of good oral absorption, bactericidal activity, and better tolerance have been used widely for the treatment of enteric fever. CLSI published revision of ciprofloxacin MIC and disk diffusion interpretative criteria in 2012 and later on in 2016. Susceptibility breakpoints for MIC value were also lowered from ≤1 to ≤0.06 µg/mL, and zone diameter increased

Table 1 Susceptibility of ciprofloxacin and pefloxacin for *Salmonella typhi*

Susceptibility	Ciprofloxacin		Pefloxacin	
	DD	MIC	DD	MIC
R	16	16	16	
S	8	8	8	
I	22 (10R; 8S; 2 UD; 2IS)	10 > 0.5–16 8 < 0.06	8S	Part A < 5 Part B < 1
		2 = 0.12–0.5 2 UD	14R	Part A ≥ 5 Part B ≥ 1

Abbreviation: DD, disk diffusion; MIC, minimum inhibitory concentration; UD, undetermined.

Table 2 Susceptibil6ity of ciprofloxacin and pefloxacin for *Salmonella paratyphi*

Susceptibility			Pefloxacin	
	DD	MIC	DD	MIC
R	–		–	
S	6	6	6	
I	4 (2R; 2S)	2 > 0.5–16 2 < 0.06	2S	Part A < 5 Part B < 1
			2R	Part A ≥ 5 Part B ≥ 1

Abbreviation: DD, disk diffusion; MIC, minimum inhibitory concentration.

Table 3 Comparison of zone diameters and MIC values for ciprofloxacin and pefloxacin

S. no	Ciprofloxacin ZOI values (in mm)	Ciprofloxacin MIC values (in mg/L)	Pefloxacin ZOI values	Pefloxacin MIC values (in mg/L)
1	26 mm	0.5	< 23	A > 5; B > 3
2	24 mm	1	< 23	A = 5; B = 3
3	29 mm	≤0.06	> 24	A = 0.1; B = 0.1
4	22 mm	16	< 23	A > 5; B > 3
5	30 mm	0.125	< 23	A > 5; B > 3
6	23 mm	1	< 23	A > 5; B > 1
7	30 mm	≤0.06	> 24	A = 0.1; B = 0.01
8	29 mm	≤0.06	> 24	A = 0.1; B = 0.01
9	24 mm	2	< 23	A > 5; B > 1
10	24 mm	1	< 23	A > 5; B > 1
11	27 mm	≤0.06	> 24	A = 0.1; B = 0.01
12	25 mm	Range not determined	< 23	A > 5; B > 3
13	24 mm	1	< 23	A > 5; B > 3
14	28 mm	≤0.06	> 24	A = 0.1; B = 0.01
15	26 mm	1	< 23	A > 5; B > 3
16	24 mm	1	< 23	A > 5; B > 3
17	23 mm	2	< 23	A > 5; B > 3
18	24 mm	Range not determined	< 23	A > 5; B > 3
19	30 mm	1	< 23	A > 5; B > 3
20	29 mm	≤0.06	> 24	A = 0.1; B = 0.01
21	28 mm	≤0.06	> 24	A = 0.1; B = 0.1
22	27 mm	≤0.06	> 24	A = 0.1; B =.01
23	24 mm	1	< 23	A > 5; B > 1
24	24 mm	2	< 23	A > 5; B > 3
25	25 mm	≤0.06	> 24	A = 0.1; B = 0.1
26	27 mm	≤0.06	> 24	A = 0.1; B = 0.01

Abbreviations: MIC, minimum inhibitory concentration; ZOI, zone of inhibition.

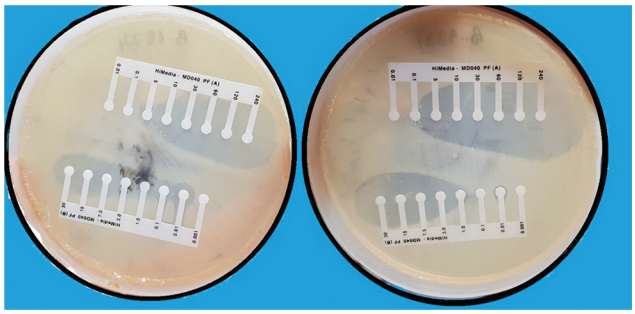

Fig. 1 MIC of pefloxacin using HICOMB gradient diffusion method. MIC, minimum inhibitory concentration.

from ≥21 to ≥31 mm. Subsequently, in 2013, ofloxacin and levofloxacin MIC interpretative criteria were included as ≤0.12 µg/mL for susceptible isolate, followed by the recommendation of the use of pefloxacin as a surrogate marker for fluoroquinolones susceptibility. Pefloxacin showed a sensitivity and specificity of 100 and 99.5%, respectively, with a positive predictive value of 94.4% for ciprofloxacin susceptibility.[8-11,13]

In our study, as shown in ►**Table 3**, 24 out of a total number of 26 isolates with intermediate susceptibility range of ciprofloxacin showed categorical agreement with pefloxacin MIC values. To measure the accuracy of commercial antimicrobial susceptibility testing, categorical agreement and essential agreement can be employed. Categorical agreement is defined as the total number of isolates tested using antimicrobial susceptibility testing that produced an MIC result and the same categorical interpretation as that of broth microdilution result. As a general rule, the performance of the commercial antimicrobial susceptibility testing should be 90% categorical agreement. Two isolates which were resistant according to pefloxacin disk diffusion test showed intermediate MIC to ciprofloxacin (MIC 0.125 and 0.5).

There is a lot of discordance between the clinical and laboratory results. This has been attributed to the complex interplay between multiple mechanisms of resistance. A single mechanism cannot be specifically responsible for the increase

in MIC and clinical failure. As guidelines for ciprofloxacin for susceptibility in *S. typhi* and *S. paratyphi A* are being frequently revised, it is ideal to have a surrogate marker which can be used to determine the resistance on the basis of simple disk diffusion method that can be done in a routine clinical microbiology laboratory in low resource setting also.[14]

In our study, we tested isolates for susceptibility to nalidixic acid, pefloxacin, and ciprofloxacin. There was a correlation between the susceptibility to ciprofloxacin and pefloxacin. However, the isolates with intermediate susceptibility had variations in terms of susceptibility to pefloxacin. This finding was corroborated with other studies that have shown that pefloxacin disk diffusion provides a better separation for ciprofloxacin susceptibility than any other disk diffusion, even better than ciprofloxacin itself.[15,16]

We also determined the MIC values for pefloxacin, and our finding suggested that pefloxacin susceptible on disk diffusion as per CLSI guidelines showed lower values for MIC using Pefloxacin HICOMB test and pefloxacin-resistant isolates showed higher MIC values. It was pivotal to perform MIC determination using HICOMB gradient diffusion test, as in routine practice sometimes it is extremely difficult to interpret the narrow range of zone of inhibition values for pefloxacin, i.e., < 23 mm for susceptible isolates and > 24 mm for resistant isolates. Further, we observed that in a situation like No. 5 isolate, ciprofloxacin ZOI = 30 mm, MIC of ciprofloxacin 0.125, and pefloxacin ZOI ≤ 23. If MIC for pefloxacin is done it shows result as A > 5, B > 3, which is an indicator that the isolate is resistant to fluoroquinolones and therapy with these can lead to treatment failure. So random testing of MIC for pefloxacin in resistant and susceptible isolates can be done in a resource compromised country to have good results. Further, it is mentioned in CLSI M100, that no one test can accurately determine all the various types of resistance to fluoroquinolones. So, may be this is a new arm in the already existing parameters of testing.[17] The limitation of our study is that the sample size is small; however, we are continuing with the study involving more centers from North India and high number of isolates. Also, instead of the commercially available test, pefloxacin MIC can be performed using in-house standardized broth microdilution method.

Source(s) of Support
Not applicable.

Conflict of Interest
None declared.

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