Current Fluoroquinolone Susceptibility Criteria for Salmonella Needs Re-evaluation

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ABSTRACT

Background

Disc diffusion technique is the routine susceptibility testing procedure for isolates of enteric fever, the most common clinical diagnosis among febrile patients in Nepal.

Objective

To evaluated the current fluoroquinolones (FQs) susceptibility criteria and nalidixic acid screening test in *Salmonella enterica* serovar Typhi and Paratyphi A.

Methods

S. Typhi and Paratyphi A strains isolated from 443 suspected enteric fever patients visiting National Public Health Laboratory (NPHL) during April through October 2008 were analyzed. All isolates were confirmed by standard microbiological procedures including serotyping. Antibiotic susceptibility testing was performed by using Kirby Bauer disc diffusion method and Clinical and Laboratory Standards Institute (CLSI) approved interpretive criteria. Agar dilution method was used to determine Minimum Inhibitory Concentration (MIC) of ciprofloxacin, ofloxacin and nalidixic acid.

Result

Out of 41 Salmonella isolates, 80.49% were nalidixic acid resistant, with S. Paratyphi A showing higher resistance rate (88.23%) compared to *S*. Typhi (75%). The difference in both MIC and zone diameter in nalidixic acid susceptible and nalidixic acid resistant isolates was found to be significant ($P < 0.001$) and decreased susceptibility to FQs was strongly correlated (sensitivity and specificity of 100%) with resistance to nalidixic acid. Regression analysis of MIC against zone diameter based on the current CLSI recommended guidelines suggests that accommodation of current susceptible and resistant MIC requires increase in the zone diameter of ciprofloxacin and ofloxacin.

Conclusion

Before using these drugs for management of enteric fever, appropriate identification of *Salmonella* isolates with reduced susceptibility to FQs is essential to limit the possible treatment failure and development of highly resistant strains. The current FQs susceptibility break point criteria for *Salmonella* need re-evaluation.

KEY WORDS

Enteric fever, Salmonella, Reduced FQ susceptibility, Nepal

INTRODUCTION

Typhoid fever caused by infection with *Salmonella enterica* serovar Typhi (S. Typhi) causes an estimated 21.6 million cases annually, with 220,000 deaths and remains a major health problem in developing countries.[1-3] *Salmonella enterica* serovar Paratyphi A (S. Paratyphi A) causes additional 5.4 million illnesses and is an emerging cause of enteric fever in South Asian countries.[2,4] During the late 1980s and early 1990s, the occurrence of multiple drug-resistant (MDR) *S.* Typhi and *S.* Paratyphi A strains, i.e. resistant to chloramphenicol, ampicillin and co-trimoxazole at a time, led to the use of fluoroquinolones (FQs) for the treatment of enteric fever.[1,5,6] The widespread use of FQs resulted in increased rate of *Salmonella enterica* strains with reduced susceptibility to these drugs.[7-9] FQs treatment, especially with short course or low-dose regimens, for *Salmonella* strains with elevated Minimum Inhibitory Concentration (MIC) though susceptible using current Clinical Laboratory Standards Institute (CLSI) breakpoint criteria, have increased the treatment failure among clinical cases.[11-12]

Disc diffusion technique is the routine procedure for

susceptibility testing since determining MIC is painstaking and rarely available in resource poor laboratories. *Salmonella* strains with reduced susceptibility to FQs are considered as susceptible to these drugs by disc diffusion according to the current CLSI interpretive criteria.[8,13] Reduced susceptibility to FQs in *Salmonella* not detected by disc diffusion tests has raised discussions regarding whether the current FQs breakpoint criteria used for *Salmonella* remain appropriate.[14,15] Use of nalidixic acid disc diffusion test has been recommended by CLSI to screen reduced susceptibility to FQs as latter is well correlated to the high-level resistance to nalidixic acid, which has been reported by several investigators.[8,13,16,17] Detection of the S. enterica serotypes with reduced susceptibility to FQs and current susceptibility criteria are being continuously evaluated.

Enteric fever is the common clinical problem in Nepal with changing resistance patterns, high level nalidixic acid resistance and increasing reports of full FQ resistance.[18-23] Using the current break point in *Salmonella* to decide the FQs therapy leads to problem of treatment failure and further fuels resistance development in developing countries where MIC is not routinely performed. Here, we report the current FQs susceptibility status and the need of reevaluation of the FQ susceptibility criteria to accommodate and differentiate susceptible and resistant strains of *S*. Typhi and *S*. Paratyphi A.

METHODS

Bacterial isolates: Forty one *Salmonella* isolates (24 *S*. Typhi and 17 *S*. Paratyphi A) recovered from 443 suspected enteric fever cases at National Public Health Laboratory (NPHL) from April through October 2008 were studied. All the isolates were subjected to both the evaluation of FQ susceptibility criteria and validation of nalidixic acid screening test. These isolates were confirmed by standard conventional methods (culture and biochemical tests) and serotyping by agglutination with specific antisera (Denka Seiken Co. Ltd., Tokyo, Japan).

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion method and CLSI recommended interpretive criteria.[13,24] *Escherichia coli* ATCC 25922 was used for quality control. Although the conformed isolates were tested for all the routine antibiotics (ampicillin 10µg, ciprofloxacin 5µg, ofloxacin 5µg, nalidixic acid 30µg, cotrimoxazole 1.25/23.75µg, tetracycline 30µg, chloramphenicol 30µg and ceftriaxone 30µg), nalidixic acid, ciprofloxacin and ofloxacin were further subjected to MIC determination (MAST, UK) . The data presented here concern with quinolones antibiotics only.

Determination of MIC: MIC of ciprofloxacin, ofloxacin and nalidixic acid was determined by agar dilution method strictly following CLSI guidelines (CLSI, 2006).[13] Briefly, a series of Mueller–Hinton agar plates, containing increasing concentrations of antibiotics (0.25 to 512µg/mL for nalidixic acid, 0.0075 to 256µg/mL for ciprofloxacin and 0.0075 to 128µg/mL for ofloxacin) were prepared. The MIC was defined as the lowest concentration of antibiotic at which there was no visible growth. E. coli ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as the quality control strains. The breakpoints defined for Enterobacteriaceae by the CLSI were considered as reference and further analysis were carried out based on these criteria. To ensure reliability and accuracy, every tests and measurement were carried out twice. Susceptibility data (with observed zone size) and MIC values of ciprofloxacin, ofloxacin and nalidixic acid were analyzed by WHONET 5.4 software. Statistical analysis was performed using SPSS 11.5. Student t-test was used to analyze difference in FQ zone of inhibition (ZOI) diameter and MIC value in nalidixic acid susceptible (NAS) and nalidixic acid resistant (NAR) isolates. Nalidixic acid validation test was carried out by using scatter plot analysis against FQs and sensitivity and specificity determination.

RESULTS

Using antimicrobial susceptibility testing by disc diffusion, 8(19.51%) isolates were found susceptible to nalidixic acid, 32(78.05%) isolates were resistant only to nalidixic acid (no ZOI) and one S. Typhi (2.4%) isolate was found fully resistant to all the three FQs tested. All the isolates were found susceptible to ciprofloxacin and ofloxacin by disc diffusion test when interpreted according to current CLSI reference (≥21mm for ciprofloxacin and ≥16mm for ofloxacin), except one isolate which was ciprofloxacin and ofloxacin resistant (12mm for ciprofloxacin and 11mm for ofloxacin). The difference in nalidixic acid resistance among *S.* Typhi and *S*. Paratyphi A isolates was statistically not significant ($P > 0.05$).

MIC and ZOI diameter of FQ: The *S*. Typhi and *S*. Paratyphi A strains resistant to nalidixic acid required higher MIC of FQs (MIC of ciprofloxacin, 0.25 to 1 µg/mL; ofloxacin, 0.25 to 2µg/mL) compared to the NAS strains (for ciprofloxacin, 7 out of 8 strains required an MIC of ≤ 0.015 and the other strain required an MIC of 0.06; for ofloxacin, 7 out of 8 strains required an MIC of ≤ 0.03 and the other strain required an MIC of 0.12). The mean MIC values and ZOI diameter along with standard deviation of FQs in two study groups (NAR and NAS isolates) have been shown in table 1 and 2.

FQ MIC and nalidixic acid resistance: Of the total isolates, 40(97.6%) were susceptible (MIC ≤ 1µg/ml), and 1 (2.4%) was resistant (MIC 16µg/ml) to ciprofloxacin. Of the 40 ciprofloxacin susceptible isolates, 32 (MIC 0.25µg/ml to 1µg/ml) were NAR and 8 (MIC 0.0075µg/ml to 0.06µg/ml) were NAS. Based on nalidixic acid susceptibility, the MIC of ciprofloxacin for susceptible isolates showed a bimodal distribution (MIC 0.25µg/mL to 1µg/mL for NAR

Table 1. MIC of FQ in nalidixic acid susceptible and resistant isolates

Agent	NAR		NAS		P value
	Mean ±SD (µg/mL)	Range (µg/mL)	Mean ±SD (µg/mL)	Range (µg/mL)	
Nalidixic acid	316±171.53	64-512	1.312±1.0288	0.5-4	<0.001
Ciprofloxacin	0.4375±0.2651	0.25-1	0.015±0.0710	0.0075-0.06	<0.001
Ofloxacin	1.0703±0.7427	0.25-2	0.0393±0.0334	0.015-0.12	<0.001

MIC, Minimum inhibitory concentration; FQ, Fluoroquinolone; NAR, Nalidixic acid resistant; NAS, Nalidixic acid resistant; SD, Standard deviation.

Table 2. ZOI diameter of FQ in nalidixic acid susceptible and resistant isolates

Agent (Disc content)	NAR		NAS		P value	Current CLSI criteria (ZOI diameter)		
	Mean ±SD	Range	Mean±SD	Range		S	I	R
Nalidixic acid (30µg)	6*	6*	23.875±1.5360	20-25	<0.001	≥19	4-18	≤13
Ciprofloxacin (5µg)	25.8125±1.875	22-28	32.625±2.2875	27-34	<0.001	≥21	26-20	≤15
Ofloxacin (5µg)	22.5625±2.090	19-25	28.875±2.3684	25-32	<0.001	≥16	13-15	≤12

*ZOI, Zone of inhibition; FQ, Fluoroquinolone; NAR, Nalidixic acid resistant; NAS, Nalidixic acid resistant; R, Resistant; I, Intyermediate, S, Susceptible; SD, Standard deviation.s

and MIC 0.0075µg/mL to 0.6µg/mL for NAS). Similarly, the MIC of ofloxacin susceptible isolates showed a bimodal distribution (MIC 0.25µg/mL to 2µg/mL for NAR and MIC 0.015µg/mL to 0.12µg/mL for NAS).

Nalidixic acid screening for reduced FQ susceptibility: Of the 33 NAR isolates, 32 were susceptible to ciprofloxacin while one was resistant. Of the 8 NAS isolates, all were susceptible to ciprofloxacin. Nalidixic acid susceptibility showed a predictive value of 100% for ciprofloxacin susceptibility, whereas nalidixic acid resistance showed a predictive value of 3.03% for ciprofloxacin resistance. This means, a substantial proportion (97%) of NAR isolates demonstrated ciprofloxacin MIC in the susceptible zone. Predictive values of nalidixic acid susceptibility against ofloxacin susceptibility and nalidixic acid resistance against ofloxacin resistance were also found similar to that of ciprofloxacin.

The relevance of using the resistance to nalidixic acid as a marker for reduced FQs susceptibility was evaluated by comparing the MIC of ciprofloxacin and ofloxacin with that of nalidixic acid for all the isolates. When a ciprofloxacin MIC of ≥ 0.125µg/mL was adopted as a breakpoint, screening for nalidixic acid resistance (MIC ≥ 32µg/mL) led to the detection of all 32 isolates with reduced ciprofloxacin susceptibility (MIC ≥ 0.125µg/mL) and none of the susceptible isolates (Figure 1, upper). Similarly, when an ofloxacin MIC of ≥ 0.25µg/mL was adopted as a breakpoint, screening for nalidixic acid resistance (MIC ≥ 32µg/mL) led to the detection of all 32 isolates with reduced ofloxacin susceptibility (MIC ≥ 0.25µg/mL) and none of the susceptible isolates (Figure 1, lower). Hence, the sensitivity and specificity of this approach was 100% for both the FQs tested.

Based on the MIC scatter-plot of ciprofloxacin and ofloxacin against ZOI diameter around 30µg nalidixic acid disc, screening for nalidixic acid resistance (ZOI diameter ≤ 13mm) led to the detection of all isolates for which the ciprofloxacin MICs were ≥ 0.125µg/mL (Figure 2, upper) and ofloxacin MIC were ≥ 0.25 (Figure 2, lower). When this MIC value was used as the breakpoint for reduced ciprofloxacin susceptibility, the sensitivity and specificity of the nalidixic acid disc screening was found 100%.

Reduced FQ susceptibility determination by single FQ disc: The applicability of 5µg ciprofloxacin and ofloxacin disc to detect reduced FQ susceptibility was also evaluated. The MICs of ciprofloxacin for 32 NAR isolates with the ciprofloxacin ZOI diameter of ≤ 28mm were ≥ 0.125µg/mL, whereas for all the NAS isolates except one (ZOI diameter = 27mm, MIC 0.06µg/mL) with ciprofloxacin ZOI diameter of ≥ 32mm, the MICs were ≤ 0.015µg/mL (Figure 3). Therefore, when an MIC of ≤ 0.125µg/mL was adopted as a breakpoint, the ciprofloxacin ZOI diameter of ≥ 32mm yielded 100% sensitivity and 87.5% specificity in screening of full ciprofloxacin susceptibility. Similarly, when an MIC of ≤ 0.25µg/mL was adopted as a breakpoint, the ofloxacin ZOI diameter of ≥ 27mm yielded 100% sensitivity and 87.5% specificity in screening of full ofloxacin susceptibility.

Regression analysis of MIC against ZOI diameter: According to regression analysis of log MIC against zone diameter (Figures 3) based on the current CLSI recommended guidelines, to accommodate a susceptible MIC of ≤ 1µg/mL, the ZOI diameter of 5µg ciprofloxacin disc for susceptible was increased to ~25mm from 21mm with corresponding increase in ZOI diameter for resistant from ≤ 15mm to ~22mm for resistant MIC of ≥ 4µg/mL. Similarly, to accommodate a susceptible MIC of ≤ 2µg/mL, the ZOI diameter of 5µg ofloxacin disc for susceptible was increased to ~22mm from16mm with corresponding increase in ZOI diameter for resistant from ≤ 12mm to ~18mm for resistant MIC of ≥ 4µg/mL.

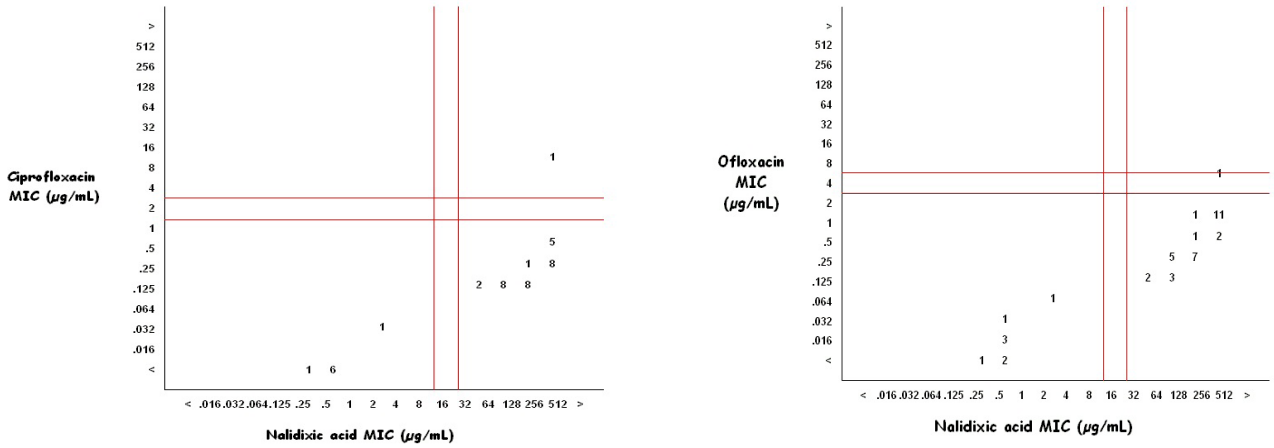

Figure 1. MIC scatter-plots for nalidixic acid against ciprofloxacin (left) and ofloxacin (right) for *Salmonella*. Current susceptibility interpretive criteria for ciprofloxacin MIC (≤1 for susceptible and ≥4 for resistant), ofloxacin MIC (≤2 for susceptible and ≥8 for resistant) and nalidixic acid MIC (≤16 for susceptible and ≥32 for resistance) are shown by parallel lines in the figure. MIC, Minimum inhibitory concentration.

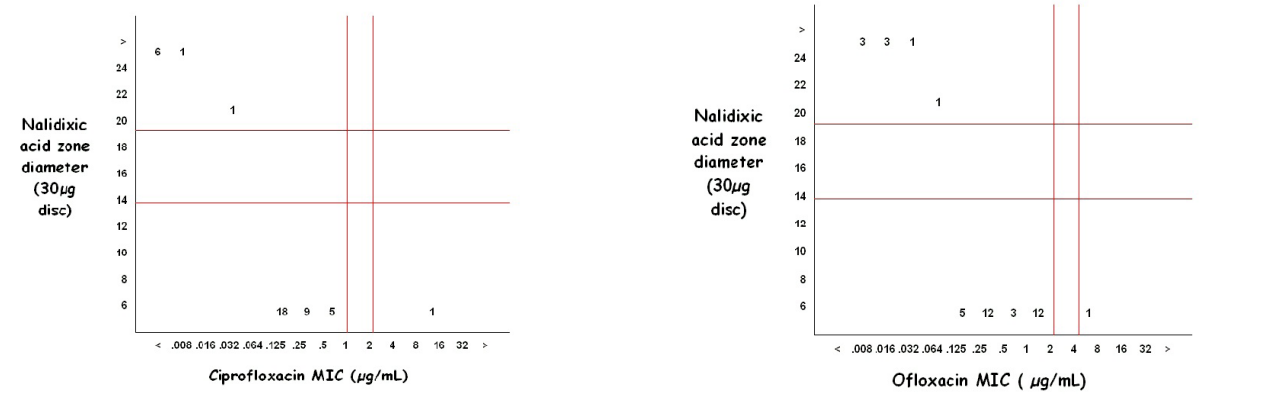

Figure 2. Scatter-plots for ZOI diameter of nalidixic acid against MIC values of ciprofloxacin (left) and ofloxacin (right): Current susceptibility interpretive criteria for ciprofloxacin MIC (≤1 for susceptible and ≥4 for resistant), ofloxacin MIC ((≤2 for susceptible and ≥8 for resistant) and nalidixic acid ZOI diameter (≤13 mm for resistance and ≥ 19 mm for susceptible) are shown by parallel lines in the figure. ZOI, Zone of inhibition; MIC, Minimum inhibitory concentration.

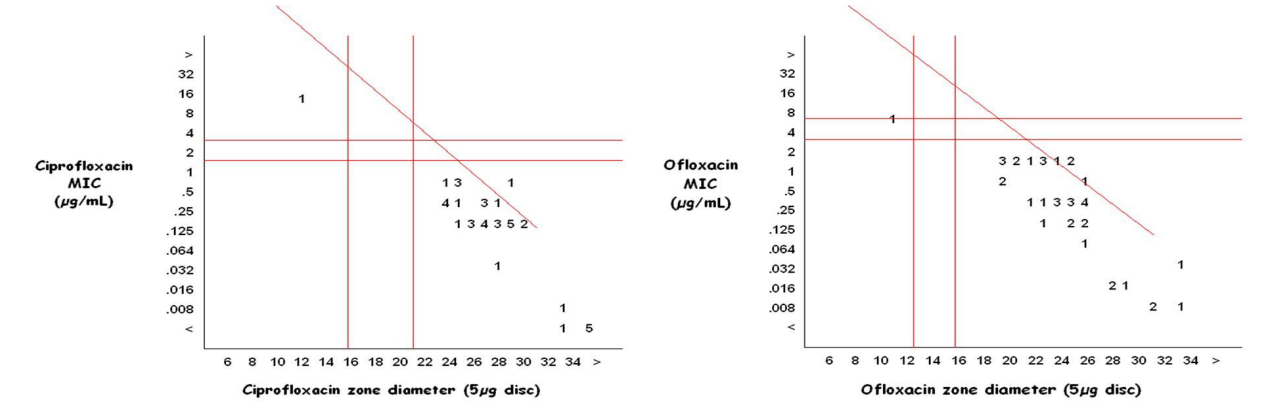

Figure 3. Scatter-plots of MIC values against ZOI diameter with regression line (ciprofloxacin left and ofloxacin right). MIC, Minimum inhibitory concentration; ZOI, Zone of inhibition.

DISCUSSION

Enteric fever remains the major diagnosis among the febrile patients attending Nepalese hospitals throughout the country and the current FQs susceptibility criteria have been found as one of the problems in the therapeutic management of enteric fever cases.[19,25] The high rate of resistance to nalidixic acid and emergence of strains with full resistance to FQs constitute a major problem in Nepal.[21-23] Appropriate identification of such strain is important

before using FQs as the first-line drugs for empirical therapy and management of enteric fever cases, however susceptibility testing generally adopted in the resource-poor laboratories of developing countries including Nepal is limited to disc diffusion technique which may not be adequate to determine reduced susceptibility to FQs.[10,11] This often requires advanced quantitative techniques such as MIC which is not available in the routine laboratories. *S.* Typhi and *S*. Paratyphi A with reduced susceptibility to FQs and resistance to nalidixic acid require higher MICs of FQs.[15] Testing for FQs susceptibility at currently accepted CLSI breakpoints fails to detect reduced sensitivity to these drugs as they are considered susceptible according to CLSI interpretive criteria. Since isolates with reduced susceptibility to FQs may become highly resistant upon sequential accumulation of mutations in topoisomerase genes, their prediction by the use of simpler screening tools implying antibiotic discs is of great value. [26-28]

We found that the nalidixic acid disc diffusion test recommended by CLSI to screen reduced susceptibility to FQs is well correlated (100%) with reduced FQ susceptibility in the Nepalese *Salmonella* isolates. The differences in the MIC values of FQs and ZOI diameters between the two study groups (NAR and NAS isolates) were statistically significant ($P < 0.001$) for all the FQs tested, supporting the association between nalidixic acid resistance and reduced FQ susceptibility. The scatter-gram correlating the MICs of ciprofloxacin and ofloxacin with nalidixic acid (Fig 1 and 2) illustrates the simultaneous presence of nalidixic acid resistance and reduced ciprofloxacin and ofloxacin susceptibility in our study population. Based on the MICs of ciprofloxacin and ZOI diameters around 30µg nalidixic acid discs, screening for nalidixic acid resistance led to the detection of all isolates (sensitivity and specificity of 100%) with reduced ciprofloxacin and ofloxacin susceptibility (MICs ≥ 0.125µg/mL) and none of the susceptible isolates.

Unfortunately, most of the resource poor laboratories and even some tertiary care hospital laboratories of Nepal are not routinely using the nalidixic acid disc screening test and/or not able to interpret the result correctly. Clinical decision to FQs therapy are often solely based on *in vitro* susceptibility of FQs, and its administration to patient have led to the treatment failure in enteric fever cases. Based on our findings, we emphasize that this simple nalidixic acid screening test should be included in the routine antibiotic susceptibility testing to screen the strains with reduced FQs susceptibility and the clinical decision to prescribe antibiotics should be based on the background of nalidixic acid resistance screening result (not merely on the single FQ susceptibility result) to prevent the possible treatment failure among enteric fever cases.

Because the therapeutic response to FQs in patients infected with NAR strains is greatly inferior compared to the response in those infected with NAS strains, several studies have suggested that the break points for the classification of Salmonella strains according to their susceptibility to FQs should be re-evaluated.[9,15,29] *S.* Typhi and *S*. Paratyphi A strains that are resistant to nalidixic acid but susceptible to FQs according to current disc susceptibility testing criteria of CLSI should be classified as non-susceptible to FQs.

Regression analysis of MIC against ZOI diameter in the present study revealed that, to accommodate a susceptible MIC of ≤ 1µg/mL, the reference ZOI diameter for susceptible needs to be increased to ≥ 26mm from 21 mm with a corresponding increase in the zone diameter for resistant isolates from 15mm to ≥ 21 mm for resistant MIC of ≥ 4µg/mL. British Society for Antimicrobial Chemotherapy (BASC) has also currently revised susceptible, intermediate and resistant breakpoints for ciprofloxacin and ofloxacin for *Salmonella*.[30] The CLSI guideline for MIC breakpoint and ZOI diameter susceptibility criteria of FQs also needs re-evaluation to accurately interpret the FQs susceptibility results (either as susceptible or non-susceptible) of Salmonella isolates which ultimately improve the management of enteric fever cases. Keeping in view of its direct relation to patient management, to minimize possible treatment failure and to limit the development of further resistance, extensive studies with diversified geographical isolates have been felt essential to establish the new susceptibility breakpoint criteria of FQs in Salmonella.

CONCLUSION

Appropriate identification of the *Salmonella* strains with reduced FQ susceptibility is a matter of concern to both laboratory personnel and physicians. This knowledge is of paramount importance when using FQs for therapeutic management of enteric fever cases since such isolates may become highly resistant upon acquisition of further mutations. The use of nalidixic acid screening test is simple but valuable tool to screen such population with reduced FQs susceptibility and every routine disc diffusion test result should be confirmed by MIC determination whenever possible. Besides, the greatly inferior therapeutic response to FQs in patients infected with *Salmonella* strains having reduced susceptibility to these drugs, and possibility of the development of highly resistant strains necessitate the re-evaluation of current FQs susceptibility break points.

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