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Nalidixic Acid Susceptibility Test for Screening *Salmonella* Isolates of Reduced Susceptibility/Higher Minimum Inhibitory Concentration to Ciprofloxacin

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Abstract
Enteric fever is the major diagnosis among febrile patients in Nepal with yearly increase in nalidixic acid resistance and reduced ciprofloxacin susceptibility among *Salmonella* isolates. This study was carried out to evaluate the validity of nalidixic acid resistance as an indicator of reduced susceptibility of *Salmonella* isolates to ciprofloxacin. In this study, 999 blood specimens collected from suspected enteric fever patients visiting B&B Hospital were processed by standard microbiological techniques. Isolates were identified by biochemical tests and serotyping. Antibiotic susceptibility test was performed by Kirby Bauer disc diffusion method and CLSI recommended interpretive criteria. MIC of ciprofloxacin was determined by agar dilution method. Isolation rate of *Salmonella* species was 6.21%. Among 62 *Salmonella* isolates, 51 were *S.* typhi, 10 were *S.* paratyphi A and one isolate was *S.* paratyphi B. Only one isolate of *S.* typhi was multi-drug resistant. Resistance to ceftriaxone, cefixime and azithromycin was nil. On disc diffusion test, 55 isolates were resistant to nalidixic acid. Fifty-seven isolates were found to have increased (≥0.125mg/ml) MIC of ciprofloxacin with the clinical and laboratory standards institute breakpoints. Nalidixic acid resistance showed a predictive value of 100% for ciprofloxacin resistance. Screening with nalidixic acid disc had a sensitivity of 100% and a specificity of 71.43% for the determination of decreased ciprofloxacin susceptibility. Before using ciprofloxacin for the treatment of enteric fever, appropriate identification of *Salmonella* isolates with reduced ciprofloxacin susceptibility is essential to limit the possible treatment failure and further development of highly resistant strains.

Key words: decreased ciprofloxacin susceptibility, enteric fever, MIC value, nalidixic acid resistance

Introduction
High prevalence of multi-drug resistant *Salmonella* typhi and *S.* paratyphi A strains during 1990s brought fluoroquinolones into picture for the management of enteric fever (Bhan *et al.* 2005, Threlfall *et al.* 1992). Subsequently, increased rates of Salmonella enterica strains with reduced susceptibility to fluoroquinolones (Molbak *et al.* 2002, Renuka *et al.* 2004) progressed into high-level resistance to these drugs (Acharya *et al.* 2009, Saha *et al.* 2006) have been reported.

Salmonella isolates with decreased ciprofloxacin susceptibility appeared susceptible by routine disc diffusion tests (Asna *et al.* 2003). Identification of such strains requires susceptibility tests, which give actual minimum inhibitory concentrations. Routine application of these tests for each strain is not convenient, and the literature suggests that resistance to nalidixic acid may be an indicator of decreased susceptibility to ciprofloxacin (Asna *et al.* 2003, Ercis *et al.* 2006, Hakanen *et al.* 1999, Kapil *et al.* 2002).

Appropriate identification of such population is a matter of concern since such isolates may result in treatment failure (Crump *et al.* 2003, Shirakawa *et al.* 2003, Threlfall *et al.* 2001) and become highly resistant

upon acquisition of further mutations (Cebrian *et al.* 2003, Gaind *et al.* 2006). This research is an attempt to evaluate nalidixic acid screening test as a tool to determine decreased susceptibility (increased MIC) of *Salmonella* isolates to ciprofloxacin.

Methodology

The study was conducted prospectively at B&B Hospital on clinically defined enteric fever patients requesting for blood culture and antibiotic susceptibility testing from April to September 2013. A total of 999 blood samples were studied which included patients of one month old to 97 years old of both sexes. Blood samples were processed by selective enrichment in Tryptone Soya Broth (TSB). Incubation and subcultures on blood agar and MacConkey agar were done as per the standard methods (Collee & Marr 1996). Suspected colonies were further processed and identified by gram's staining followed by required biochemical tests and confirmed by group and type specific *Salmonella* antisera (Denka Seiken Co. Ltd., Tokyo, Japan). All the *Salmonella* spp. isolated from blood samples were subjected for antibiotic susceptibility test by Kirby Bauer disc diffusion method in compliance with CLSI guidelines on Mueller Hinton agar plates using ampicillin (10 µg), azithromycin (15 µg), cefixime (5 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg) and nalidixic acid (30 µg) (HiMedia Laboratories Pvt. Ltd., Mumbai, India). *Escherichia coli* ATCC 25922 was used for quality control. MIC of ciprofloxacin was determined by agar dilution method following CLSI guidelines (CLSI, 2013). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains. All the data collected were analyzed using statistical software SPSS version 16.0. The chi-square ($\chi 2$) test was used to determine the significance of differences. Nalidixic acid susceptibility data (with observed inhibition zone diameter) and MIC values of ciprofloxacin were analyzed by WHONET 5.6 software. Nalidixic acid validation test was carried out by using scatter-plot analysis against ciprofloxacin, and sensitivity and specificity determination. The sensitivity and specificity in determining nalidixic acid resistance by disc testing, with respect to MIC values of ciprofloxacin, were calculated using formulae: % Sensitivity=true resistant / (true resistant + false sensitive)×100, and % Specificity=true sensitive / (false resistant +true sensitive)×100. The procedures followed were in accordance with the ethical standards of the Ethical Review Board at Institutional Review Committee (IRC) on human experimentation at B&B Hospital.

Results and Discussion

Among 999 patients studied during 6 months period, 62 (6.21%) were confirmed as enteric fever cases. Of 62 isolates, 51 (82.26%) were identified as *S.* Typhi, 10 (16.13%) as *S.* Paratyphi A, and one isolate (1.61%) as *S.* Paratyphi B. S. Paratyphi C was not isolated. The distribution of these serotypes also varied among different age groups (Table 1).

Table 1. Distribution of the suspected febrile cases and enteric fever cases in different age group

S.N.	Age group (years)	Total suspected febrile cases	Total enteric fever pathogens		
			Isolated (%)	*S.* Typhi isolates	*S.* Paratyphi isolate
1.	<5	101	1 (0.99)	1	-
2.	5-18	200	28 (14)	26	2
3.	19-45	420	30 (7.14)	22	8
4.	>45	278	3 (1.08)	2	1
	Total	999	62 (6.21)	51	11

Table 1 displays the distribution of the suspected febrile cases and enteric fever cases in different age groups. Although the overall growth rate was only 6.21%, the percentage of growth in the age group 5-18 years was 14% followed by 7.14% in the age group 19-45 years. The difference in enteric fever cases by age group was statistically significant ($P < 0.05$).

Male preponderances were seen in infections caused by both the organisms (male: female ratio = 32:19 for S. typhi and 6:5 for S. Paratyphi, i.e., 19:12 for all *Salmonella* isolates) (Table 1). The gender-wise difference was statistically insignificant ($P>0.05$).

The most number of suspected febrile cases were observed in the months of July and August and culture positive cases were also greater at those months as 6.25% and 7.00% respectively (Fig. 1).

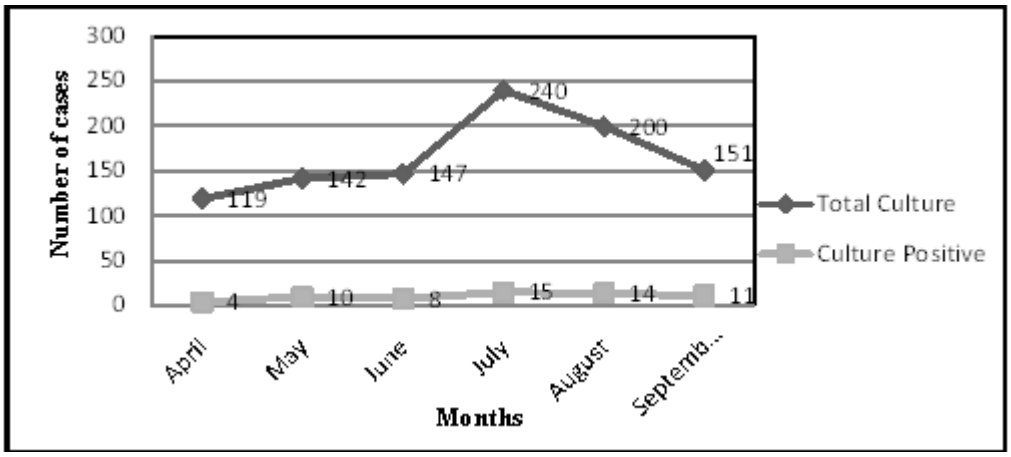

Fig. 1. Pattern of blood culture positive cases at different months

Fig. 2 shows antimicrobial resistance pattern of *Salmonella* isolates. S. Typhi showed 100% susceptibility to azithromycin, cefixime and ceftriaxone followed by 98% susceptibility to chloramphenicol and cotrimoxazole. Resistance to ampicillin and ciprofloxacin was found to be 31.4% and 80.4% respectively. Moreover, one isolate (2.0%) among S. Typhi was found to be multidrug resistant (MDR) being simultaneously resistant to first line drugs like ampicillin, chloramphenicol and cotrimoxazole (ACCo).

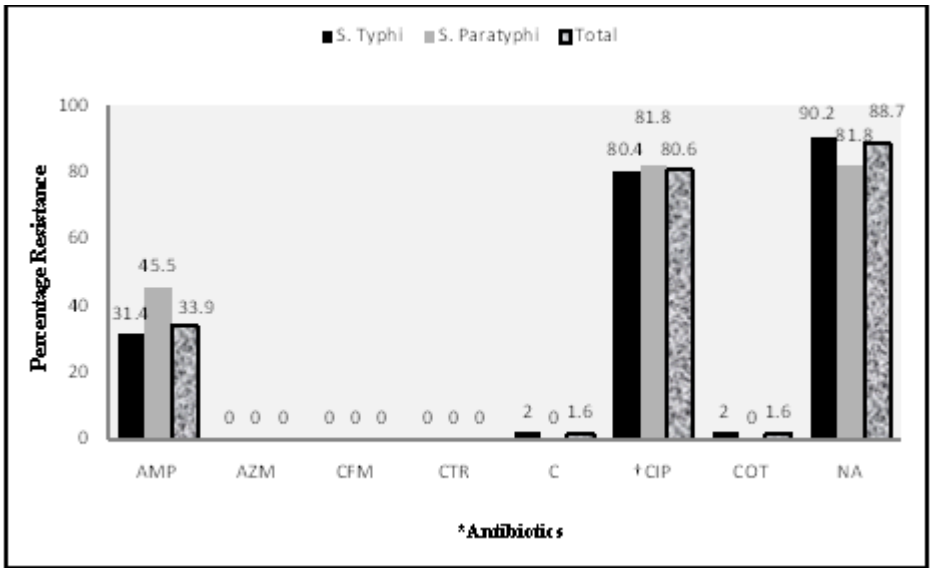

Fig. 2. Antibiotic resistance pattern in *S. typhi* and *S. paratyphi*

* AMP (Ampicillin); AZM (Azithromycin); CFM (Cefixime); CTR (Ceftriaxone); C (Chloramphenicol); CIP (Ciprofloxacin); COT (Cotrimoxazole); NA (Nalidixic acid).
† Intermediate resistance observed with ciprofloxacin has been illustrated as resistance in the figure.

Similarly, S. Paratyphi demonstrated 100% susceptibile to azithromycin, cefixime, ceftriaxone, chloramphenicol and cotrimoxazole. Resistance of isolates towards ampicillin and ciprofloxacin was found to be 45.5% and 81.8% respectively. By contrast with S. Typhi, none of S. paratyphi were MDR.

The overall nalidixic acid resistance rate was quite high (88.7%). S. Typhi strains showed even higher (90.2%) resistance towards nalidixic acid than S. Paratyphi (81.8%) The difference in nalidixic acid resistance among S. Typhi and S. Paratyphi A, however, was statistically insignificant (P>0.05).

MIC value of ciprofloxacin was determined for 62 isolates, 55 NAR and 7 NAS *Salmonella* isolates (Figure 3). The 5 (8.07%) isolates had MIC <0.125 mg/ml, and 57 (91.93%) were found to have increased MIC (≥0.125 mg/ml) of ciprofloxacin. Among 55 NAR isolates (zone of inhibition ≤13mm) by disc testing, 32 showed full resistance to ciprofloxacin, 23 exhibited intermediate resistance and none were fully susceptible. Of the 7 NAS isolates (zone of inhibition ≥19mm), 5 were fully susceptible to ciprofloxacin and two exhibited reduced ciprofloxacin susceptibility. Thus, screening with 30µg nalidixic acid discs had a sensitivity of 100% and a specificity of 71.43% for determination of *Salmonella* isolates with decreased susceptibility (increased MIC) to ciprofloxacin.

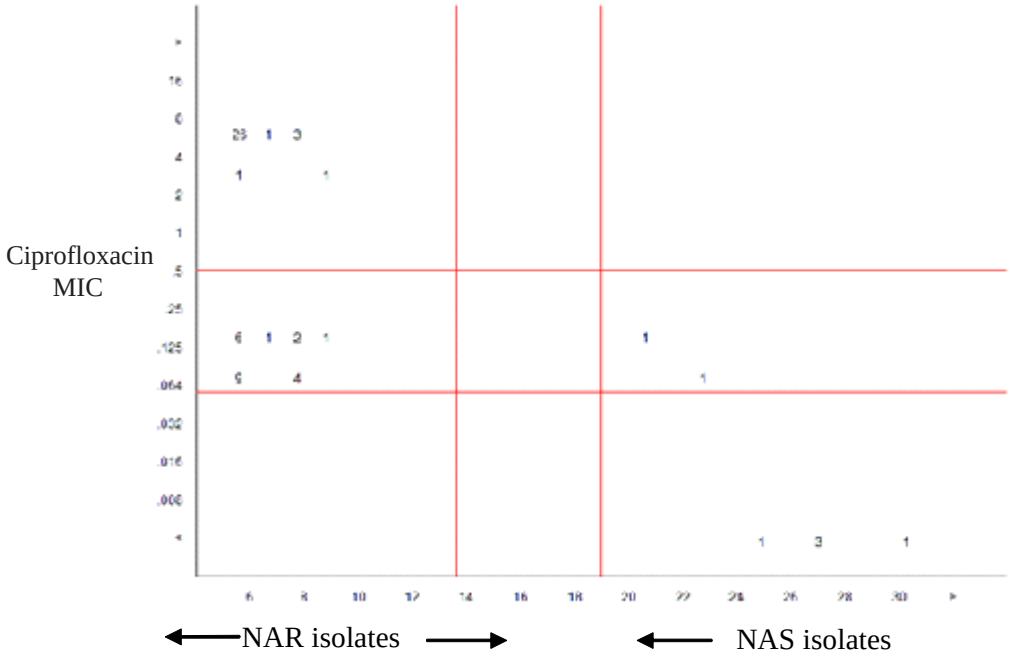

Fig.3. Scatter-plot for MIC values of ciprofloxacin against inhibition zone diameter of nalidixic acid (output from WHONET 5.6 after analysis): Current susceptibility interpretive criteria for ciprofloxacin MIC (d"0.064 for susceptible and e"1 for resistant) and nalidixic acid inhibition zone diameter (d"13 mm for resistance and e" 19 mm for susceptible) are shown by parallel lines in the figure.

Growth positive rate of *Salmonella* species in this study was 6.21%. Similar positive rates have also been reported by Acharya (2008), and Shrestha (2011) which was 9.1% and 4.69% respectively. In contrast, high positive rate (19.28%) has been reported by Kunwor (2007) from Kathmandu. Relatively low culture positive rate observed in this study might be due to the use of antibiotics prior to blood collection for culture. S. typhi (82.26%) was more commonly isolated than S. paratyphi (17.74%) in the present study. Based on the previous reports, an estimated one case of paratyphoid fever occurs for every four cases of typhoid fever (Crump *et al*. 2004). This estimate was seen in this study.

Despite the majority, 420 (42.0%) of the suspected cases fell in age group 19-45 years, higher percentage of enteric fever cases (14%) were observed among 5-18 years consisting of only 200 samples. In a recent study carried out in five Asian countries, 75% of the

growths were from the age group of 5-15 years (Ochiai *et al*. 2008). The particular age group seems to be more vulnerable to exposure as they may not be having enough care for their foods and potable drinking water due to their busier schedule than that of other age groups. *Salmonella* infection has been found predominantly in males (61.3%) in this study. Previous reports (Acharya 2008, Khanal *et al.* 2007) from Nepal have also shown higher prevalence of Salmonella infection in males than in females. More outdoor exposure of males has been given the possible reason for higher positive rate among them.

The maximum occurrence of *Salmonella* infection was observed in July and August, the months that had the temperature and rainfall relatively high. This may be possibly due to the sewage-mediated contamination of water samples during the rainy seasons. Faecal contamination of urban water supplies in Nepal has been reported. An outbreak of *S.* Typhi infecting 5,936 people in Bharatpur in 2002 was traced to the municipal water supply (Lewis *et al*. 2005).

In this study, resistance to traditional first-line antibiotics such as chloramphenicol and co-trimoxazole has significantly decreased to 1.6% in comparison to previous report by Khanal *et al*. (2007) who reported 27.2% resistance to each antibiotic. But fluoroquinolones which are so frequently used nowadays in empirical treatment of enteric fever showed higher resistance pattern to ciprofloxacin. All strains with reduced susceptibility to ciprofloxacin were fully susceptible to cephalosporins such as ceftriaxone and cefixime. There have been sporadic reports of high-level resistance to ceftriaxone among S. Typhi and S. Paratyphi A (Saha *et al*. 2006). Although these strains are very rare. Pokharel *et al*. (2006) has reported ceftriaxone resistance and ESBL production by S. Paratyphi A in Nepal.

In this study, overall nalidixic acid resistance rate was quite high (88.7%). S. Typhi strains showed even higher (90.2%) resistance towards nalidixic acid than *S.* Paratyphi (81.8%) which is in agreement with the finding of Acharya (2008). In addition to the nalidixic acid resistance, this study found resistance of *Salmonella* strains to ampicillin (31.4% S. Typhi and 45.5% S. Paratyphi A). In a study done in Chitwan, Acharya *et al*. (2012) reported very high percentage of ampicillin-resistant isolates (70.6% S. Typhi and 78.3% S. Paratyphi A). It indicates that ampicillin will have very limited value in treating enteric fever. Only one strain of S. Typhi met the conventional definition of multi-drug resistant (MDR) being simultaneously resistant to ampicillin, chloramphenicol and cotrimoxazole (ACCo). An interesting observation in the present study is that MDR in S. Typhi has come down to 1.96% in comparison to previous reports given by Acharya *et al*. (2012); Khanal *et al*. (2007); and Pokharel *et al.* (2006) which were found to be 16.66%, 26.5% and 5.18% MDR isolates respectively.

The findings of this study indicate a remarkable decline in the number of MDR isolates. This was accompanied by an increase in non-MDR isolates, though the majority of these (88.7%) was NAR and showed reduced susceptibility to ciprofloxacin. These findings are most likely due to decreased prescribing of traditional antimicrobial agents and an increasing reliance on ciprofloxacin as the first-line treatment for S. Typhi and S. Paratyphi. Reports from most of the countries (Maskey *et al*. 2008, Saha *et al*. 2002, Weill *et al*. 2007) showed the incidence of MDR isolates appears to have decreased with significant increase in the isolates with reduced susceptibility to fluoroquinolones.

According to current CLSI recommendation, susceptible, intermediate and resistant breakpoints for ciprofloxacin among *Salmonella* spp. are ≤0.064 µg/ml, 0.125-0.5 µg/ml and ≥1 µg/ml (respective inhibition zone diameter to 5µg ciprofloxacin are ≥31mm, 21-30mm and ≤20mm) (CLSI 2013). Analysis of the MIC values of ciprofloxacin in this study found that 5 (8.1%) isolates were fully susceptible, 25 (40.3%) were intermediately resistance and 32 (51.6%) exhibited full resistant to ciprofloxacin.

The relevance of using the resistance to nalidixic acid as a marker for reduced ciprofloxacin susceptibility was evaluated by comparing the MIC of ciprofloxacin with inhibition zone diameter of nalidixic acid for all the isolates. Among 55 NAR isolates (zone of inhibition ≤13mm), MIC values of ciprofloxacin were found to be 0.125 µg/ml, 0.25 µg/ml, 4 µg/ml and 8 µg/ml for 13, 10, 2 and 30 isolates respectively. The 23 of the NAR isolates had decreased ciprofloxacin susceptibility and 32 isolates were found to exhibit high-level resistance to ciprofloxacin. Although 7 NAR isolates appeared susceptible to ciprofloxacin by disc

diffusion method when interpreted according to current CLSI reference (zone of inhibition ≥31mm), they showed increased MIC to ciprofloxacin (MIC ≥0.125 µg/ml) which demonstrated that all NAR isolates were with reduced susceptibility to ciprofloxacin.

In the present study, the reduced susceptibility to ciprofloxacin in S. Typhi and S. Paratyphi was strongly correlated with resistance to nalidixic acid. The scatter-plot correlating the MIC of ciprofloxacin with inhibition zone diameter of nalidixic acid (Figure 3) illustrated the simultaneous presence of nalidixic acid resistance and reduced ciprofloxacin susceptibility. Similar to this finding Lynch *et al.* (2009) reported from the United States that the NARST isolates, among which 97% had decreased ciprofloxacin susceptibility.

In this study, out of 7 NAS isolates, 5 (71.43%) were fully susceptible to ciprofloxacin (MIC < 0.125mg/ml) and two exhibited reduced ciprofloxacin susceptibility while all of the NAR isolates were less-susceptible or resistant to ciprofloxacin (MIC ≥ 0.125µg/ml). Thus, screening with 30mg nalidixic acid disc had a sensitivity of 100% and a specificity of 71.43% for determination of decreased susceptibility to ciprofloxacin.

In contrast to high sensitivity of nalidixic acid screening test, the emergence of new quinolone resistance pattern in *S. enterica* which are susceptible to nalidixic acid but exhibited reduced susceptibility to ciprofloxacin (Hakanen *et al*. 2005) was also reported from South Asia. The increase of such strains (3.23% in the present study) may threaten the value of the nalidixic acid disc test to screen for reduced fluoroquinolone susceptibility in salmonellae.

Salmonella strains with decreased susceptibility to fluoroquinolones are of concern due to the increasing number of treatment failures in invasive salmonellosis (Hakanen *et al*. 1999). In many tropical countries, including Nepal and Indian subcontinent, there is widespread availability and uncontrolled use of fluoroquinolones. As strains that are already exhibiting decreased susceptibility to ciprofloxacin may require fewer exposures to fluoroquinolones to develop high-level resistance to ciprofloxacin than the strains that are fully ciprofloxacin susceptible (Cebrian *et al*. 2003, Gaind *et al*. 2006). The use of fluoroquinolones as first-line drugs for empirical therapy and management of enteric fever in areas where these strains are endemic is questionable and requires an urgent review. Low exposure to fluoroquinolones reducing the selective pressure on a large bacterial population would definitely lessen the likelihood of selecting mutants (Gaind *et al*. 2006).

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