Susceptibility to Fluoroquinolones among Salmonella enterica Serovars in Blood Culture

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ABSTRACT

Objectives: The study was designed to analyze the antibiotic susceptibility pattern of fluoroquinolones among Salmonella enterica.

Methods: A cross-sectional study was carried out at National Public Health Laboratory, Kathmandu. Blood samples were collected from suspected enteric fever patients and cultured in BACTEC standard/10 Aerobic/F culture vials. Isolates obtained from the vials with bacterial growth were identified by standard procedure. Serotyping of the identified isolates Salmonella enterica was done. An antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method and results were interpreted according to Clinical Laboratory Standards Institute (CLSI 2014) guidelines.

Results: Among 404 samples, 17 (4.2%) were positive for Salmonella enterica in which 9 (52.9%) were Salmonella Typhi and 8 (47.1%) were Salmonella Paratyphi A. All the Salmonella isolates showed resistance to nalidixic acid and ampicillin and showed sensitivity to ceftriaxone and chloramphenicol. No multi-drug resistant isolates were identified in this study. All isolates of Salmonella Typhi and Salmonella Paratyphi A showed the reduced susceptibility to ciprofloxacin and ofloxacin.

Conclusion: It is concluded that fluoroquinolones cannot be considered as the drug of choice for the treatment of Salmonella infections due to their high level of reduced susceptibility and resistance to fluoroquinolones and third generation cephalosporin antibiotics like ceftriaxone remains better choice of drugs against fluoroquinolone-resistant Salmonella Typhi and Paratyphi.

Key words: Enteric fever, Salmonella enterica, fluoroquinolones, nalidixic acid resistant

INTRODUCTION

Enteric fever is a serious bloodstream infection caused by Salmonella enterica serovars Typhi and Paratyphi A and is an important cause of morbidity and mortality (Britto et al 2020; Maes et al 2020). The global estimated cases of enteric fever and deaths due to enteric fever in 2017 are 14.3 million and 135.9 thousand respectively, whereas Salmonella enterica serotype Typhi caused 76·3% of cases of enteric fever (GBD 2017). Enteric fever has been a public health concern in Nepal, with S. Typhi and S. Paratyphi A consistently being regularly isolated from the blood of febrile patients in Kathmandu Valley since the early 1990s (Maskey et al 2008; Murdoch et al 2004). In developing countries like Nepal, the mainstay therapy is antibiotics to prevent the complications associated with enteric fever illness and death of the patients. With the introduction of chloramphenicol for the treatment of typhoid fever in 1948, often fatal disease was transformed into a readily treatable condition and the cases has been reduced to less than 1% from about 30%, however a major setback occurred with the emergence of resistance to chloramphenicol and other antimicrobial agents (Parry et al 2002).

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Antibiotics recommended by World Health Organization (WHO) for enteric fever treatment are chloramphenicol, ampicillin and cotrimoxazole (trimethoprim-sulfamethoxazole), fluoroquinolones, third-generation cephalosporins (ceftriaxone, cefixime) and azithromycin for the treatment of enteric fever (WHO 2003). However, the reduced susceptibility of *Salmonella enterica* isolates to commonly used antibiotics continues to be a major problem for effective therapy of enteric fever, prolonging the duration of fever and leaving patients at risk of further complications (Bhetwal et al 2017; Zellweger et al 2017). Due to the emergence of multidrug resistance (MDR) strains of *Salmonella*, the antibiotic treatment of enteric fever with the first line antibiotics chloramphenicol, ampicillin, and cotrimoxazole has been affected (Eng et al. 2015), which led to the use of fluoroquinolones, mainly ciprofloxacin, and third generation cephalosporin (Bhutta 2006; Bhan et al 2005; Pokharel et al 2009)

Fluoroquinolones, such as ciprofloxacin and ofloxacin, have become a mainstay for treating severe *Salmonella* infections (Sjölund-Karlsson et al 2014). However, strains of *Salmonella* with increased levels of resistance to fluoroquinolones have been reported in South Asia (Browne et al. 2020) and in Nepal (Acharya et al 2012; Bhetwal et al 2017; Maskey at al 2008). Nalidixic acid resistance (NAR) is a marker for predicting decreased susceptibility (low-level of resistance) to ciprofloxacin among *S. enterica* serovar Typhi and Paratyphi, and also an indicator of treatment failure to ciprofloxacin (Acharya et al 2012; Khademi et al 2020; Rudresh et al 2015).

In the developing countries like Nepal where minimum inhibitory concentration of antibiotics is not routinely determined in laboratories, fluoroquinolones especially ciprofloxacin is still used for the treatment. There would be the possibility of treatment failure of infections with *S.* Typhi and *S.* Paratyphi A strains with reduced fluoroquinolone susceptibility (Crump et al 2004; Woods et al 2006). This study was thus designed with the objectives to determine the reduced susceptibility pattern of *Salmonella* isolates towards fluoroquinolones and to find out the minimum inhibitory concentration (MIC) value of the ciprofloxacin and ofloxacin which might help to know the effective drug dose to be used for the treatment of the typhoid fever.

METHODS

The cross-sectional study was carried out at National Public Health Laboratory (NPHL), Kathmandu, Nepal from June 2016 to November 2016. This study was conducted on clinically defined enteric fever patients of all age groups of both sexes who visited NPHL requesting for blood culture and susceptibility testing. The ethical approval for this study was obtained from Nepal Health Research Council, Kathmandu, Nepal (Approval no. 363/2016).

A total of 404 blood samples from patients suspected of enteric fever were included in the study after obtaining the consent and details on clinical history, age and sex of the individual were recorded. The exclusion criteria for samples were improper labeling, insufficient blood volume, inappropriate collection and transport, and samples from patients with prior antibiotic therapy within 1 week. About 3-5 mL of blood from patients was collected and aseptically inoculated into BACTEC standard/10 Aerobic/F culture vials. The inoculated culture vials were immediately transported to the laboratory and incubated in BACTEC fluorescent series instruments. Incubation was continued for 7 days until growth indication was obtained in BACTEC. The culture bottles were observed daily for indication of microbial growth. The growth was indicated by the red alarm in the BACTEC machine. The aliquot from vials with growth of bacteria were subcultured on MacConkey agar (MA) and blood agar (BA) plates. The final subculture for visually negative culture bottles was done after 7 days of incubation.

The isolated colonies of bacteria obtained on MA and BA were analysed for the identification as *Salmonella* spp by Gram staining and biochemical tests. Various biochemical tests- catalase test, oxidase test, sulphide indole motility (SIM) test, methyl red test, Voges Proskauer test, triple sugar iron (TSI) test, citrate test and urease test were performed for Gram negative rods (Cheesbrough 2012; WHO 2003). Serotyping of bacteria identified as *Salmonella enterica* was also done to confirm the isolates with antisera by observing the agglutination reaction between antigen and antibodies. For serotyping, O, H and Vi antigen of Denka – Seiken company Ltd, Japan was used. (add few details of serotyping). After complete identification, isolates were preserved in tryptic soy broth with 25% glycerol at -70 ^{o}C.

Antibiotic susceptibility tests (AST) of the identified bacteria were performed by modified Kirby Bauer disc diffusion method on Mueller Hinton Agar (MHA) plate following CLSI guideline 2014. Minimum inhibitory concentration (MIC) of ciprofloxacin and ofloxacin were determined by broth dilution method the concentrations of 0.125 to 512 µg/mL for ciprofloxacin and 0.125 to 512 µg/mL for ofloxacin, and following the guidelines of CLSI

(2014). *Escherichia coli* ATCC 25922 was used as the quality control strain.

RESULTS

Out of 404 cases, only 17 (4.2%) cases were found to be culture positive for *S. enterica*. Among the *Salmonella* isolates, 9(52.9%) were *S*. Typhi and 8 (47.1%) were *S*. Paratyphi.

Growth of *Salmonella enterica* was not obtained in the samples of patients of age greater than 40 years. The highest percentage of growth was seen in the age group 11-20 and 21-30 years (29.4%). Greater number of male patients were infected with *Salmonella* than females as shown in Table 1.

Ceftriaxone and chloramphenicol were 100% effective to all isolates followed by cotrimoxazole. However, organisms showed resistance towards quinolone group antibiotics as nalidixic acid was 100% ineffective followed by ciprofloxacin (47.1%) as shown in Table 2.

The MIC breakpoint values of ciprofloxacin used for interpretation were ≤0.06 µg/mL, 0.125-0.5 µg/mL and ≥1 µg/mL as sensitive, intermediate and resistant respectively according to CLSI guideline 2014. In this study, the highest MIC value of ciprofloxacin for Nalidixic Acid Resistant isolates was 1 µg/mL and the lowest was 0.25 µg/mL as shown in Table 3, whereas the highest MIC value of ofloxacin was 1 µg/mL and the lowest was 0.5 µg/mL as shown in Table 4.

Table 1. Distribution of the serotypes of *Salmonella* in different age group and gender

Age Group (years)	Number of enteric fever cases with growth of *Salmonella enterica*				Total
	Male		Female		
	S. Typhi	*S*. Paratyphi	*S*. Typhi	*S*. Paratyphi	
0-10	2	2	-	-	4
11-20	1	2	1	1	5
21-30	3	1	1	-	5
31-40	-	2	1	-	3
Total	6	7	3	1	17

Table 2: Antibiotic susceptibility pattern of *Salmonella* serotypes (concentration of antibiotics)

Antibiotic Used	Antibiotic Susceptibility Pattern					
	Susceptible		Intermediate		Resistant	
	Number	Percent (%)	Number	Percent (%)	Number	Percent (%)
Nalidixic Acid (30 mcg)	-	-	-	-	17	100
Ciprofloxacin (5 mcg)	1	5.9	8	47.1	8	47.1
Ceftriaxone (30 mcg)	17	100	-	-	-	-
Ampicillin (10 mcg)	-	-	-	-	17	100
Cotrimoxazole (25 mcg)	15	88.3	2	11.8	-	-
Azithromycin (15 mcg)	8	47.1	4	23.5	5	29.4
Chloramphenicol (30 mcg)	17	100	-	-	-	-

Ofloxacin (5 mcg)	11	64.7	6	35.3	-	-
Cefixime (5 mcg)	14	82.4	3	17.6	-	-
Amikacin (30 mcg)	16	94.1	1	5.9	-	-

Table 3: MIC of Ciprofloxacin susceptibility pattern of Nalidixic Acid resistant *Salmonella* serovars for *S.* Typhi and *S.* Paratyphi.

MIC (µg/mL)	*S.* Typhi (N=9)	Sensitivity pattern towards Ciprofloxacin	*S.* Paratyphi A (N=8)	Sensitivity pattern towards Ciprofloxacin	MIC Breakpoints
≤0.015	-	Sensitive (N=0)	-	sensitive (N=0)	Sensitive ≤0.06 µg/mL
0.03	-		-		
0.06	-		-		
0.125	-	Intermediate (N=8) 88.9%	-	Intermediate (N=7) 87.5%	Intermediate 0.125-0.5 µg/mL
0.25	6		5		
0.5	2		2		
1	1	Resistant (N=1) 11.1%	1	Resistant (N=1) 12.5%	Resistant ≥1 µg/mL
2	-		-		

Table 4: MIC of Ofloxacin susceptibility pattern of nalidixic acid resistant *Salmonella* serovars for *S.* Typhi and *S.* Paratyphi.

MIC (µg/mL)	*S.* Typhi (N=9)	Sensitivity pattern towards Ofloxacin	*S.* Paratyphi A (N=8)	Sensitivity pattern towards Ofloxacin	MIC Breakpoints
≤0.015	-	Sensitive (N=0)	-	Sensitive (N=0)	Sensitive ≤0.12 µg/mL
0.03	-		-		
0.06	-		-		
0.125	-				
0.25	5	Intermediate (N=9) 100%	-	Intermediate (N=8) 100%	Intermediate 0.25-1 µg/mL
0.5	3		6		
1	1		2		
2	-	Resistant (N=0)	-	Resistant (N=0)	Resistant ≥2 µg/mL

DISCUSSION

Salmonella enterica in the blood culture of suspected patients was found only 4.2% and similar culture positivity results have also been reported in other studies from Nepal (Bhetwal et al 2017; Shrestha et al 2016). However, the relatively higher growth rate was reported by Sharma et al (2006) and Khanal et al (2007) as 6.9% and 5.1% respectively. Rewrite these sentences. Growth rate of the present study was low but even lower growth rate has been reported as 2% in Raza et el 2012 and 2.3% among children in Kathmandu by Maskey et al 2008. Decrease in growth might be the result of antibiotic therapy even in milder cases of fever (Malla et al 2005; Khanal et al 2007).

The positive cases were higher (76.5%) in male patients than in female (23.5%). This study result was comparable to the study done in Lalitpur by Pandey et al (2015), which reported 70.2% male and 29.7% female positive cases. Previous studies from Nepal also have shown higher prevalence of enteric fever in males than in females (Sharma et al 2003; Shakya et al 2008; Prajapati et al 2008). This study showed a higher prevalence of enteric fever among males as the number of samples was higher in male. This gender wise difference in the prevalence of enteric fever may be due to sample size (male:female = 1.23:1) and their relatively more outdoor activities exposing them to the source of infection. Majority of the cases were of the age group between 11-20 years and 21-30 years, followed by 0-10 years and 31 -40 years. Similar types of the result have been reported in the study carried out by Agrawal et al 2014 with majority 14% of cases in the age group 5-18 years. These age groups include school and college going children. The possible causes for enteric fever being common in these age groups include their mobility, consumption of unhygienic food and water in street vendors, schools and colleges (Walson et al 2001).(references)

Two serotypes i.e. *S*. Typhi (52.9%) and *S*. Paratyphi A (47.1%) were identified in this study. This result was comparatively similar with the study result of Raza et al (2012) as *S*. Typhi (66.7%) and *S*. Paratyphi A (33.3%) and in the study done by Gurung et al (2017), 54% *S*. Typhi and 46% *S*. Paratyphi A.

The isolates were tested against ten antibiotic discs for performing the antibiotic susceptibility testing. Among the isolated *S*. Typhi showed 100% sensitivity towards chloramphenicol and cotrimoxazole while *S.* Paratyphi A showed 100% sensitivity to chloramphenicol and 75% to cotrimoxazole which was similar to the study done by Amatya et al (2007) and Joshi et al (2011). In this study, though chloramphenicol was found susceptible to all the isolates, it is not recommended as a drug of choice due to its side effects. Ceftriaxone should be recommended only if the first and second line antibiotics failed to evoke a satisfactory response or if the isolate is resistant to nalidixic acid. So, it should be a last line drug during empirical therapy and also shows the high sensitivity to chloramphenicol and cotrimoxazole (Manchanda et al 2006; Neupane et al 2008; Prajapati et al 2008; Sharma et al 2007; Acharya et al 2012). Ofloxacin and cefixime was shown to be 77.8% sensitive to *S*. Typhi followed by azithromycin (55.6%), whereas in *S*. Paratyphi, cefixime shows 87.5% sensitivity followed by ofloxacin (50%) and azithromycin (37.5%). As in this study ampicillin was 100% resistant to all the isolates, and similar report of high percentage of ampicillin-resistant isolates (70.6% S. Typhi and 78.3% S. Paratyphi A) was shown in a study done in Chitwan by Acharya et al (2012).

In this study, all isolates were found to be 100% nalidixic acid resistant (NAR), which was higher in comparison to other studies. This trend of higher nalidixic acid resistance was also found in a study conducted in Kathmandu by Shirakawa et al (2006) and Agrawal et al (2014) in which nalidixic acid resistant in *S*. Typhi were 73.3% and 90.2% and *S*. Paratyphi were 94.9% and 81.8% respectively. In developing countries, the high resistance of nalidixic acid is often due to self- medication (Mincey and Parkulo 2001), the suboptimal quality of antimicrobial drugs, and poor community and patient hygiene (Walson et al 2001).

This study also showed high frequency of ciprofloxacin resistant isolates with 55.6% in *S*. Typhi, 37.5% in *S*. Paratyphi A. High resistance to ciprofloxacin was also observed in study of Poudel et al 2014 with 31.3% in *S*. Typhi, 4% in *S*. Paratyphi A. This increased resistance reflects the overuse of ciprofloxacin in the treatment of typhoid, as well as in other unrelated infections. Incomplete treatment may also be a factor contributing to development of resistance. Third generation cephalosporin, ceftriaxone showed 100% susceptibility for both *S*. Typhi and *S*. Paratyphi A strains in present study. Similarly, a study conducted by Sharma et al 2003 in Dhulikhel hospital also reported 100% efficiency of ceftriaxone to both strains. Ceftriaxone remains as the last line of drug against infections with ciprofloxacin resistant *Salmonella* when it is resistant to other first line drugs (Bhatia et al 2007; Raza et al 2012).

According to CLSI (2014), susceptible, intermediate and resistant breakpoints for ciprofloxacin among *Salmonella*

spp. are ≤0.06 µg/mL, 0.125-0.5 µg/mL and ≥1 µg/mL (respective inhibition zone diameter to 5 µg ciprofloxacin are ≥31 mm, 21-30 mm and ≤20 mm). Similarly, for ofloxacin are ≤0.12 µg/mL, 0.25-1 µg/mL and ≥2 µg/mL (respective inhibition zone diameter to 5 µg ofloxacin are ≥16 mm, 13-15 mm and ≤12 mm) (CLSI 2014). In this study, only one NAS strain was ciprofloxacin sensitive by disc diffusion method but none of the strain was susceptible in MIC (MIC ≤0.06 µg/mL). Among 17 NAR isolates, 8 (47.1%) isolates were resistant by disc diffusion but only 2 (11.8%) were found to be resistant by MIC test (MIC 1 µg/mL). However, 15 (88.2%) showed the reduced susceptibility towards ciprofloxacin (MIC value 0.125-0.5 µg/mL). The reduced susceptibility to ciprofloxacin in *S*. Typhi and *S*. Paratyphi A was strongly correlated with resistance to nalidixic acid. Similarly, by performing MIC test towards ofloxacin to all NAS isolates, 11(64.71%) isolates were sensitive by disc diffusion but none of the isolate was sensitive as MIC value ≤0.12 µg/mL. All 17(100%) showed the reduced susceptibility towards ofloxacin (MIC value 0.25-1 µg/mL). Similarly, in the study done by Acharya et al 2012, it was reported that nalidixic acid disc diffusion recommended by CLSI (2014) to screen reduced susceptibility to fluoroquinolones was well correlated with reduced fluoroquinolones susceptibility in the *Salmonella* isolates.

Many studies done in Kathmandu have also reported the cases of enteric fever treated with fluoroquinolones with prolonged time or treatment failure. The MIC of ciprofloxacin and ofloxacin of such strains is steadily increasing, although the MIC values were still below CLSI (2014) recommended breakpoint (≤1 and ≥4 µg/mL) (Adhikari et al 2012; Nagshetty et al 2010; Rudresh et al 2015). However, it is not clear whether fluoroquinolones can still be used as first-line drugs for the treatment of typhoid fever, and if used whether this has any adverse impact on clinical outcomes other than treatment failure such as development of complications and morbidity assessed in terms of total duration of illness. In such a scenario, this present study was carried out to determine the infection of NARST isolates and the effectiveness of fluoroquinolones against the isolates. Because of the rising rates of quinolone resistance, there is a clear need to identify improved strategies for treating typhoid fever as highly resistant organisms may be isolated in near future (WHO 2003). The drug of choice for the treatment of enteric fever is ceftriaxone, Chloramphinicol, Amikacin however Cefixime and Cotrimoxazole can be used for the treatment with antibiotic susceptibility test.

CONCLUSION

The prevalence of *Salmonella enterica* serovar Typhi was found to be relatively higher than *Salmonella enterica* serovar Paratyphi A among significant growth obtained from blood culture. Though fluoroquinolones are the first choice for the treatment of enteric fever, high level of reduced susceptibility and resistance to fluoroquinolones (*S*. Typhi and *S*. Paratyphi - resistant to nalidixic acid) were observed, raising question in the efficacy of fluoroquinolones used for the treatment of enteric fever. Therefore, the third generation cephalosporin antibiotics like ceftriaxone might be a better choice for treatment against fluoroquinolone resistant *Salmonella* Typhi and Paratyphi. Hence, this study suggests that nalidixic acid susceptibility test by disc diffusion method can be used as the screening test to determine decreased susceptibility of *Salmonella* strains to fluoroquinolones and MIC determination becomes mandatory for NAR *Salmonella* strains.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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