Prevalence Of Salmonella Species With Nalidixic Acid Resistance In The Enteric Fever Patients Of Nepal

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***Abstract*—Enteric fever is one of the most common public health problems in Nepal. The progressive increase in antibiotic resistance among pathogens in developing countries is becoming a critical area of concern globally. The present study was carried out at Everest Hospital, Kathmandu, Nepal, with an objective to determine the prevalence of enteric fever and to analyze the status of antimicrobial resistance pattern of Salmonella enterica serovar Typhi and Paratyphi isolated from blood specimens. Blood samples collected were used for culture in Brain Heart Infusion broth. The broth cultures showing turbidity were subcultured, and antibiotic susceptibility testing was performed. Identification of the isolates was done by standard microbiological techniques, and antibiotic susceptibility testing was performed by modified Kirby Bauer disc diffusion method following clinical and laboratory standard guidelines (CLSI). A total of 692 blood samples were collected during April, 2015 to September, 2015. Among them 40(5.8%) samples were found to be culture positive whereas 652 (94.2%) cases were culture negative. Out of 40 isolates; 22 (55%) were S. Typhi and 18 (45%) were S. Paratyphi A, and the prevalence was higher in male and in the age group of 11-20 years. Most effective antibiotics were chloramphenicol and cotrimoxazole. Among the 22 S. Typhi isolates, 21 (95.5%) were nalidixic acid resistant (NAR). Out of 18 S. Paratyphi A isolates, 16 (88.9%) were nalidixic acid resistant. Among 21 nalidixic acid resistant (NAR) S. Typhi, only 2 isolates were found to be resistant to ofloxacin and only 11 isolates were found to be sensitive to ciprofloxacin. Similarly, out of 16 isolates of S. Paratyphi A resistant to nalidixic acid. It is concluded that higher rate of nalidixic acid resistance as well as emerging resistant pattern to other fluroquinolone antibiotics suggests us to follow correct treatment regimens and good infection control practices.**

***Keywords*—Enteric fever, Salmonella Typhi, S. Paratyphi, fluroquinolone antibiotics, nalidixic acid resistant (NAR) INTRODUCTION**

Enteric fever is a common term to encompass two similar clinical illnesses, caused by different serotypes of the bacterium *Salmonella enterica* [1], including *Salmonella enterica* serotype Typhi and *Salmonella* enterica serotype Paratyphi A, B and C [2]. Enteric fever is continued to be one of the most common infectious disease in many of the developing world, especially in Asia and the global situation has worsen since the emergence of multi-drug resistance in 1989 [3].

It is the most common clinical diagnosis among febrile patients presenting to hospital in Nepal [4]. It is locally known as Bhisam jwaro or Mayade jwaro in Nepal [4]. Recently, 162 cases of enteric fever per 100,000 local residents have been reported in Nepal. But the prevalence of *Salmonella enteric* B and C infection in Nepal is not reported so far [2]. Hence, the present study was undertaken to isolate and identify *salmonella species* from clinical samples and to know their antibiotic susceptibility pattern in an urban tertiary care center.

Chloramphenical was the standard treatment for enteric fever from the 1950s until the development and spread of multidrug resistant (MDR; defined as resistance to all first line antibiotics: Chloramphenical, Ampicillin and Co-trimoxazole) *S.* Typhi and *S.* Paratyphi A in the early 1990s. Subsequently Fluoroquinolones, in particularly Ciprofloxacin and the third generation Cephalosporins became choice for the treatment of enteric fever [5]. However, the widespread use of Fluoroquinolones led to emergence of *S.* Typhi and Paratyphi strains with reduced susceptibility or resistance to Fluoroquinolones [6]. In recent years, workers in several countries have reported the treatment failure after administration of ciprofloxacin therapy in patients with enteric fever caused by *Salmonella enterica* with reduced susceptibility to ciprofloxacin. These three nalidixic acid resistant *S.* Typhi (NARST) require higher

concentration of fluoroquinolones for inhibition. Based on the antimicrobial susceptibility testing, the isolates can be broadly as sensitive, only nalidixic acid resistant, and multidrug resistant with nalidixic acid resistant (MDR-NAR) [7].

Thus, isolation and identification of *S.* Typhi and Paratyphi, and determination of its antibiotic susceptibility pattern are crucial for several reasons like selecting appropriate antimicrobial agencies, improving prognosis of patients, declaring the acquisition of resistance in pathogens and reducing expenditure on overall hospital costs. This study helps in the treatment of patients more efficiently and generating surveillance data which will be useful to formulate an effective antibiotic policy in the hospital.

MATERIALS AND METHODS

A descriptive cross sectional study was carried out at Everest Hospital, Kathmandu, Nepal from April 2015 to December 2015 on patients having a febrile episode lasting for ≥ 3 days attending at Everest Hospital. Cases included in the study were patients defined by physicians as probable case of enteric fever with fever (38°C and above) that had lasted for at least three days and showing clinical signs and symptoms of enteric fever. A total of 692 blood samples from patients following case definition of suspected enteric fever were included in the study. Samples with improper labeling, insufficient blood volume, inappropriate collection, and transport were rejected.

Blood collection

5-10 ml blood samples of suspected enteric fever patients were collected by venipuncture method and processed for laboratory testing. Details on clinical history, age, sex, and previous antibiotic administrations of the individual were recorded by using the questionnaire.

Isolation and identification of Salmonella serotype

The culture bottles were incubated at 37°C. Incubation was continued for 7 days unless the visible growth was obtained. The blind subculture for visually negative culture bottle was done after 7 days of incubation. The day of collection of sample was defined as the first day in this study. After that, macroscopic examination of broth culture was performed by examining daily for visual evidence of microbial growth in broth culture. The blood culture showing turbidity was subculture in MacConkey agar and was incubated at 37ºc for 24 hours and examined for the growth of non-lactose fermenting colonies and gram stain and various biochemical tests as recommended by CLSI was done for the identification of bacteria [8].

Antimicrobial susceptibility testing

The identified bacteria was reported and antibiotic sensitivity testing was done by Kirby-Bauer method of disk diffusion technique as recommended by NCCLS using Mueller Hinton agar (MHA) [8]. Briefly, three to five well isolated colonies of bacteria of the same morphological types were selected from the MacConkey agar plate to a tube containing 5ml of nutrient broth and was incubated at 37°C (usually 2 to 6 hours) until it achieved the McFarland tube number 0.5. In case of overgrowth, the broth was diluted with sterile physiological saline to match with McFarland tube number 0.5. A sterile cotton swab was dipped into the broth and the swab was rotated several times and pressed firmly on the inner side wall of the tube above the fluid level to remove excess inoculums from the swab. Then the dried surface of a MHA plate was inoculated by streaking the swab over the entire agar surface three times. Finally the inoculum was left to dry for a few minutes at room temperature with the lid closed. Then the antimicrobial disks were placed on the surface of the prior inoculated agar plate such that there was 25mm distance from disk to disk. Then they were incubated aerobically at 37ºC overnight. After overnight incubation, the diameter of zone of inhibition (ZOI) was measured. It is then compared with standard chart to determine bacterial susceptibility toward different antimicrobial agents in terms of ‘sensitive’, 'Intermediate', and resistant. The antibiotics used were ampicilline, Cefixime, Ceftriaxzone, chloramphenicol, ciprofloxacine, Cotrimoxazole, gentamycin, Nalidixic acid, and ofloxacin from Himedia Company, Mumbai, India.

Data analysis

All the data were collected and statistically analyzed using Microsoft Excel and SPSS version 20. P-values < 0.05 were considered statistically significant.

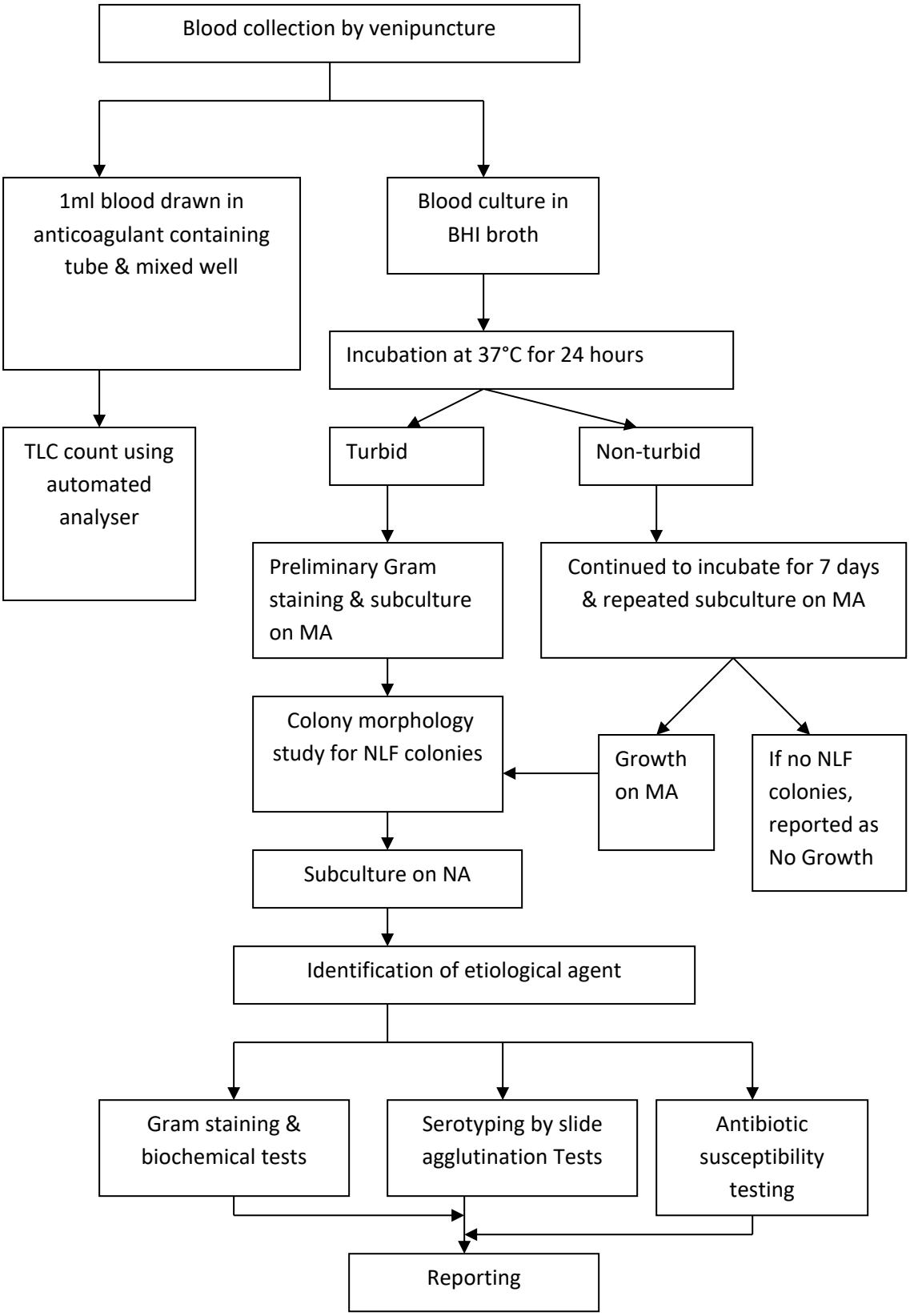

Figure1: Flow chart for isolation and identification of *Salmonella species* from blood culture.

RESULT

A total no of 692 patient suspected with enteric fever where included in the study conducted during July to December 2016 at Everest hospital Kathmandu, Nepal. Among them, 371 patients were male and 321patients were female.

Distribution of febrile illness patients

Among the total 692 cases, the highest number of cases (210) were from age group 21-30 years, followed by 150 cases from the age group 11-20 years.

Age group (Years)	Total number of patients			
	Male	Female	Total	Percentage (%)
Up to 10	43	30	73	10.5
11-20	79	71	150	21.7
21-30	106	104	210	30.3
31-40	73	47	120	17.3
41-50	40	30	70	10.1
51-60	21	20	41	5.9
61-70	6	12	18	2.6
71-80	1	7	8	1.2
81-90	2	0	2	0.3
Total	371	321	692	100

Table 1: Age/sex wise distribution of febrile illness patients. In this study, the highest percentage of growth was seen in age group 21-30 years (30.3%), followed by age group 11-20 years (21.7%), 31-40 years (17.3%) and 41-50years (10.1%).

Distribution of enteric fever patients.

Age group (Years)	Number of positive culture				P- value
	Male	Female	Total	Growth (%)	
Up to 10	5(1.3%)	1(0.3%)	6	8.2	
11-20	9(2.4%)	3(0.9%)	12	8	
21-30	5(1.3%)	5(1.6%)	10	4.8	
31-40	1(0.3%)	3(0.9%)	4	3.3	
41-50	0	4(1.3%)	4	5.7	
51-60	1(0.3%)	1(0.3%)	2	4.9	(P>0. 05)
61-70	1(0.3%)	0	1	5.5	
71-80	0	0	0	0	
81-90	1(0.3%)	0	1	50	
Total	23(6.2%)	17(5.3%)	40		

Table 2: Age and sex wise distribution of enteric fever patients. From 692 cases investigated, 40 cases (5.7%) were found to be culture positive and 652 cases were (94.3%) were found to be culture negative for enteric fever patients.

Proportion of *S.* Typhi and *S.* Paratyphi A in enteric fever patients

A total of 40 isolates, distribution of *S.* Typhi and *S.* Paratyphi A are 22 (55%) and 18 (45%) respectively. Involvement of *S*. Paratyphi B and C in the infection was absent.

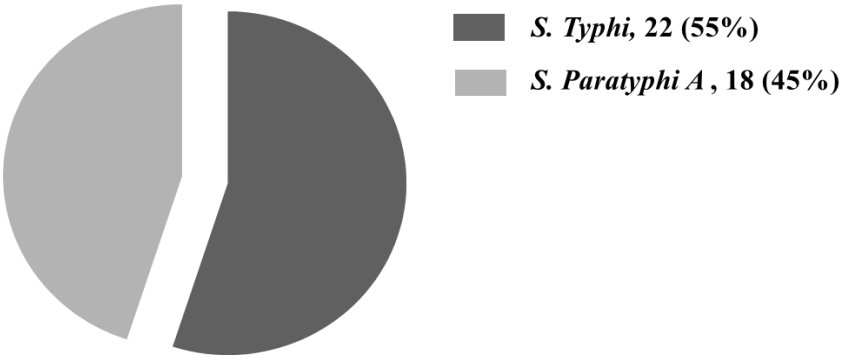

Figure 2: Distribution of *S.* Typhi and *S.* Paratyphi A in enteric fever patients. A total of 40 isolates, distribution of S. Typhi and S. Paratyphi A are 22 (55%) and 18 (45%) respectively.

Age Group (Years)	Distribution of salmonella species						Growth (%)
	Male			Female			
	ST	SPA	Total	ST	SPA	Total	
Up to 10	3	2	5	1	-	1	15
11-20	6	3	9	2	1	3	30
21-30	1	4	5	1	4	5	25
31-40	1	-	1	-	3	3	10
41-50	-	-	-	3	1	4	10
51-60	1	-	1	1	-	1	5
61-70	1	-	1	-	-	-	2.5
71-80	-	-	-	-	-	-	0
81-90	1	-	1	-	-	-	2.5
Total	14	9	23	8	9	17	**100**

Table 3: Age and sex wise distribution of *S.* Typhi and *S*. Paratyphi A in enteric fever patients. Out of 40 isolates highest isolates were from age group 11-30 years irrespective of genders and only 1growth in male of age group 81-90 years. (ST, *Salmonella* Typhi; SPA, *Salmonella* Paratyphi A)

Antibiotic susceptibility pattern of the *Salmonella* Typhi.

All the 40 isolates obtained were tested for antibiotic susceptibility pattern using 10 different antibiotics. Among 22 isolates of *Salmonella* Typhi, Cotrimoxazole and Chloramphenicol were found to be 100% effective in all isolates. It was followed by Ceftriaxone with 90.9% and gentamycin with 86.4% effective. The resistant pattern was high towards Nalidixic acid (95.5%) followed by Amoxycillin (90.9%).

Classes of Antibiotics	Antibiotics used	Antibiotics susceptibility pattern					
		Sensitive		Intermediate		Resistance	
		No	%	No	%	No	%
Penicillin	Amoxycillin	2	9.1	-	-	20	90.9
Macrolides	Azithromycin	11	50.0	9	40.9	2	9.1
Cephalosporin	Cefixime	16	72.7	2	9.1	4	18.2
	Ceftriaxone	20	90.9	1	4.5	1	4.5
Chloramphenicol	Chloramphenicol	22	100	-	-	-	-
Sulphonamides	Cotrimoxazole	22	100	-	-	-	-
Aminoglycosides	Gentamycin	19	86.4	-	-	3	13.6
Fluoroquinolones	Nalidixic acid	1	4.5	-	-	21	95.5
	Ciprofloxacin	11	50.0	2	9.1	9	40.9
	Ofloxacin	15	68.2	5	22.7	2	9.1

Table 4: Antibiotic susceptibility pattern of *Salmonella* Typhi. Among 22 isolates of *Salmonella* Typhi, Cotrimoxazole and Chloramphenicol were found to be100% effective in all isolates. It was followed by Ceftriaxone with 90.9% and gentamycin with 86.4% effective. The resistant pattern was high towards Nalidixic acid (95.5%) followed by Amoxycillin (90.9%).

Antimicrobial susceptibilitypattern of *S.* Paratyphi A.

All the 18 isolates obtained were tested for antibiotic susceptibility pattern using 10 different antibiotics. Among 18 isolates of *Salmonella* Paratyphi A, Chloramphenicol and Cotrimoxazole was found to be 100% effective in all isolates. It was followed by Gentamycin with 94.4% susceptibility. Sixteen (88.9%) isolates were found to be resistant towards Nalidixic acid and Amoxycillin. The percentage of isolates resistant to cefixime was 22.2%.

Classification of antibiotics	Antibiotics used	Antibiotics susceptibility pattern					
		Sensitive		Intermediate		Resistance	
		No	%	No	%	No	%
Penicillin	Amoxycillin	1	5.6	1	5.6	16	88.9
Macrolides	Azithromycin	14	77.8	1	5.6	3	16.7
Cephalosporin	Cefixime	13	72.2	1	5.6	4	22.2
	Ceftriaxone	15	83.3	2	11.1	1	5.6
Chloramphenicol	Chloramphenicol	18	100	-	-	-	-
Sulphonamides	Cotrimoxazole	18	100	-	-	-	-
Aminoglycosides	Gentamycin	17	94.4	1	5.6	-	-
Fluoroquinolones	Nalidixic acid	2	11.1	-		16	88.9
	Ciprofloxacin	13	72.2	4	22.2	1	5.6
	Ofloxacin	17	94.4	-	-	1	5.6

Table 5: Antibiotic susceptibility pattern of *Salmonella* Paratyphi A. Among 18 isolates of *Salmonella* Paratyphi A, Chloramphenicol and Cotrimoxazole was found to be 100% effective in all isolates. It was followed by Gentamycin with 94.4% susceptibility. Sixteen (88.9%) isolates were found to be resistant towards Nalidixic acid and Amoxycillin. The percentage of isolates resistant to cefixime was 22.2%.

DISCUSSION

Enteric fever is a major health problem in developing countries attributed to poor sanitary and hygienic conditions. It is attributed to rapid population growth and unplanned urbanization, inadequate and improper waste disposal, lack of potable water supply. In Nepal, sources of many drinking water (piped, natural taps/spouts and wells) are heavily contaminated by fecal materials. In the cities, contamination of water is resulted due to cross contamination with sewage. Therefore, the disease has been remained endemic with outbreaks occurring time and again [8]. Emergence of drug resistance in the organism poses great challenge to physicians. Physicians, therefore, should always be aware of the antibiotic sensitivity/resistance profile of the organism in a given community for the rational use of antibiotics [5].

This study was done with an objective to compare the disease burden and the antibiotic resistance among enteric fever patients within a stipulated time period in Everest Hospital, kathmandu, Nepal. Six hundred and ninety two blood samples were collected from patients visiting Everest hospital and processed for culture using standardized clinical and microbiological methods. Among the 692 blood specimen collected from febrile illness patients who had fever for >3days, 40 cases (5.8%) were found to be culture positive for enteric fever whereas 652 (94.2%) cases were culture negative (Table 2). Almost similar results have been also reported by Acharya et al., 2012 [8] and Sharma et al., 2012 [9] which show 7.6% and 8.9% of entric fever patients respectively. According to those findings the growth rate of present study was somewhat comparitively low but positive rate even lower (2%) than present findings has also been reported [10]. In contrast to present study, relatively higher growth rate (23.1%) has been reported by Amatya et al., 2007 [11]. Although blood culture remains a gold standard for diagnosis, the poor sensitivity of this method has been acknowledged. In a large study conducted with 21847 samples with febrile episode in five Asian countries, S. Typhi was isolated only 2% by blood culture[12]. According to Bhan et al., 2005 in a study conducted in rural setting in Nepal, the incidence of enteric fever was found to be only 6.5 per 1000 cases [3]. Amatya et al., 2007 from Kathmandu reported positive rate of 23.1% [11] whereas Maskey et al., 2008 [13] from Kathmandu reported 18.8%.

The low incidence of positive cases was probably due to the availability of over-the-counter antimicrobials and the likely possibility that self-medication before presentation for physician's care reduced the sensitivity of blood cultures. To increase the chance of isolating a pathogen, it is usually recommended that at least two specimen (multiple blood collected at different times) should be cultured [14] but our reliance upon a single blood culture for diagnosis has an effect on the observed rate of enteric fever which may undoubtedly be an underestimate.

Among a total of 692 febrile cases, 371 (53.6%) were male whereas 321 (46.4%) cases were female (Table 1). The total number of culture positive cases i.e. enteric fever cases were 23 (57.5%) and 17 (42.5%) in male and female (1.3:1) respectively. Previous studies from Nepal have shown higher prevalence of enteric fever in males than in females[4, 9, 15]. Similarly male to female ratio has been reported by Bhattarai et al., 2003 (1.2:1) [16] and Ansari et al., 2002 (1.3:1) [17] in the Kathmandu valley. The male preponderance seen could be due to

their relatively more outdoor activities exposing them to the source of infection. This sex wise difference in the prevalence of enteric fever might be due to relatively small sample size. Besides, other behavioral and socio-economic factors may also play important role.

The blood culture positive rate was highest 12 (30%) in the age group 11-20 years consisting of 150 (21.7%) samples and blood sample was highest with 210 (30.3%) samples in the age group 21-30 years. These age groups include school and college going children. The possible cause for enteric fever being common in water in street vendors, school and colleges [8]. The incidence of enteric fever was found highest in children and young adults between 5 and 19 years old [18]. Similar type of the result have been reported, in the study carried out by Parande et al., 2011 with majority 40.7% were in the age group 11-20 years in Solapur, India [19].

In the same way, out of 40 blood culture positive cases, 22 (55%) were *S.* Typhi and 18 (45%) were *S.* Paratyphi A; indicating higher prevalence of typhoid cases than paratyphoid cases. *S.* Paratyphi B and C were not isolated during the study period. This finding was similar to other studies carried out in Nepal. The percentage of *S.* Typhi and *S.* Paratyphi A was found to be 69.8% and 29.0% respectively by Thapa,1991 [20] ; 63.6% and 35.0% respectively by Shrestha, 1996 [21]. Based on report from different part of the world including India, Pakistan, China and Vietnam, *S.* Paratyphi A is now an emerging cause of enteric fever [2]. A study conducted in Nepal showed that more than half (65.4 %) were caused by *S*. Paratyphi A whereas remaining (34.6 %) by *S.* Typhi [22].

In the present study, the isolates obtained from the blood culture bottles were tested against ten antibiotic discs for antibiotic susceptibility testing. Among the isolated *S.* Typhi, 100% susceptibility was found with the antibiotics chloramphenicol and cotrimoxazole. Ceftriaxone and gentamycin were also found to be the antibiotics of choices with 90.9% and 86.4% of effectivity respectively (Table 4). Altogether 9.1% *S.* Typhi isolates were resistant to azithromycin and ofloxacin each and 4.5% were found resistant to ceftriaxone. In the same way, resistant rate for nalidixic acid in *S.* Typhi was found to be 92.5% (Table 4). This result is supported by Sharma et al., 2006 which stated the maximum i.e. 95.5% isolates were resistant towards nalidixic acid [15]. Another study conducted by Parajuli, 2014, it was found that 85.7% of *S.* Typhi isolated were resistant to Nalidixic acid [22].

Consequently; Chloramphenicol, Cotrimoxazole, and gentamycin were found to be predominantly effective in all isolates of *S.* Paratyphi A, however Nalidixic acid and Amoxycillin were found to be resistant. This finding is also fully supported by a study from India in 2005 which shows that 100% of *S.* Paratyphi A were resistant to Nalidixic acid [23]. The reason for high resistance of blood pathogenic bacteria to antimicrobial agents in developing countries is often due to self- medication, the suboptimal quality of antimicrobial drugs, poor community, increased use of antibiotics, and patient hygiene. However, it is not clear whether fluoroquinolones can still be used as first-line drug 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 scenario, the present study was undertaken to determine the infection by NARST isolates and the effectiveness of ciprofloxacin against these isolates.

CONCLUSION

Nepal is one of the typhoid endemic countries in Asia and is a hot bed for enteric fever. This study reveals that the prevalance of enteric fever is found to be most common among males than in females, and highly observed among adlosent patients. The prevalance of *Salmonela* Typhi is relatively higher than *Salmonella* Paratyphi A among enteric fever patients.

This study also explores the fact that the fluoroquinolone drugs which have been used as the first choice for the treatment of enteric fever is predominantly resistant to enteric fever. And the third generation cephalosporin drugs like chloromphenicol is the better choice of drug against fluoroquinolone resistant *Salmonella* Typhi and Paratyphi.

AUTHORSHIP CONTRIBUTION

Santosh Kumar Gupta: Conceptualization, Writing, Supervision. Ram Chandra Panday: Investigation, visualization, data analysis, writing of draft, fund acquisition. Jay Prakash Sah: Writing, editing, reviewing. Ganesh Dhakal: Validation, Reviewing. Dipak mahaseth: Writing, paper searching, Khushbu Shah: writing and paper searching.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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