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Enteric fever caused by *Salmonella enterica* serovar paratyphi A: An emerging health concern in Nepal

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Enteric fever is an invasive life-threatening systemic disease caused by the *Salmonella enterica* human-adapted serovars typhi and paratyphi. Increased incidence of infection with *S. enterica* serovar paratyphi A poses a significant health concern in some areas of the world. In this study, the incidence of enteric fever confirmed by isolation of *Salmonella* paratypi A or S. typhi from blood cultures of patients presenting with clinical symptoms was 5.1%. Of the total isolates, 64.13% were *S*. paratyphi A, and 35.87% were *Salmonella* typhi. All isolates were susceptible to amoxicillin, ceftriaxone and cefixime. Conventional antibiotics (ampicillin, cloramphenicol and co-trimoxazole) showed 100% sensitivity rate towards *S*. paratyphi A and 96.9% towards *S*. typhi. Overall nalidixic acid-resistance (NAR) rate was extremely high (92.39%). Nalidixic acid resistant (MIC ≥ 32 µg/ml) *S.* paratyphi A showed increased MICs of the fluoroquinolone than nalidixic acid resistant *S*. typhi ranges from 0.125-8 µg/ml with ciprofloxacin and 0.25-4 µg/ml with levofloxacin and was statistically significant (p≤0.001). Immunization with currently available vaccines against typhoid fever does not provide cross protection against paratyphoid fever. This may contribute to the emergence of paratyphoid fever as the major cause of enteric fever in Nepal and possibly other geographical locations.

Key words: Enteric fever, *Salmonella* paratyphi A, *Salmonella* typhi, fluoroquinolones (FQs), Nepal.

INTRODUCTION

Salmonella enterica serovars typhi (*S*. typhi) and paratyphi (*S*. paratyphi) A, B, C are human restricted bacterial pathogens that cause related systemic disease, collectively called enteric fever, remains a common febrile illness in the developing world including the Indian subcontinent, Southeast Asia, Africa, and, to a lesser extent, South America, with poor standard of hygiene and sanitation (Kathryn et al., 2007; Crump et al., 2010; Kariuki et al., 2004). Current estimates from World Health Organization (WHO) suggest that the global burden of typhoid fever is approximately 21 million cases annually with more than 2,20,000 deaths, and that paratyphoid

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fever causes an additional 5.4 million cases (Crump et al., 2004).

In recent years, the incidence of infection with *S*. paratyphi A is elevated in some regions of the globe (Particularly in South-east Asia). It is accountable for up to 50% of all enteric fever cases, causing more asymptomatic infection than *S*. typhi (Woods et al., 2006; Neupane et al., 2010; Mahapatra et al., 2016). In developed countries, enteric fever is a sporadic disease that occurs mainly in returned travelers from the endemic areas (Lee et al., 2004). In recent years, countries like Japan and United States of America have already experienced increase incidence of *Salmonella* paratyphi A in returned traveler from various endemic regions (Judd et al., 2015; Katanami et al., 2016). In Australia, Among 810 *S.* paratyphi A isolated between1985-2010, 547 isolates originated from India, Indonesia, Bangladesh, Pakistan, Nepal, Cambodia, Thailand, Philippines, Papua New Guinea and Lebanon (Commons et al., 2012). In another study carried out in Sydney, 8 *S.* paratyphi A infections were detected during the period January-June 2011 and the patients were predominantly associated with travels to the Indian subcontinent (Blackstock et al., 2012). Recently, three cases of *S*. paratyphi A infection have been reported in French traveler after trekking in Nepal during monsoon season (Jean et al., 2016). A recent outbreak of *S.* paratyphi A in India, Combodia suggests how this neglected tropical diseases rapidly spread in various geographical region of the globe (Verma et al., 2016; Laura et al., 2015).

In Nepal, higher isolation rate of *S.* paratyphi A during summer has been documented in various studies and has become one of the most common culture isolates from patients with febrile illness (Woods et al., 2006; Acharya et al., 2011; Shirakawa et al., 2006). In a retrospective study, 288 out of 541 blood culture samples from patients with enteric fever collected in Tribhuvan University Teaching Hospital, Kathmandu between January and September, 2004 were serotyped as *S.* Paratyphi A (Pokharel et al., 2006).

FQs like ciprofloxacin and ofloxacin, are relatively inexpensive and well tolerated, considered as the most selected groups of antimicrobial for the treatment of uncomplicated enteric fever in adults (Chuang et al., 2009). Unfortunately, outbreaks of *S.* paratyphi A strains that were resistant to nalidixic acid (the prototype quinolone, which is used for in vitro screening tests), accomplished reduced susceptibility to the FQs have been reported subsequently in India, Pakistan, China and South East Asia (Parry et al., 2002; Chuang et al., 2009; Hakanen et al., 1999; Threlfall et al., 1999) and infections with elevated MICs to FQs have been related with the treatment failure and increases disease severity (Hakanen et al., 1999; Renuka et al., 2004).

Third generation cephalosporin is associated with higher cure rates in the FQs resistant patients (WHO, 2003). However, resistance in third generation cephalosporin from the different parts of the world in *S*. paratyphi A, is an ever increasing problem, and is a cause of serious concern for the treatment of enteric fever (Pokharel et al., 2006; Vincet et al., 2008; Nashwan et al., 2008; Morita et al., 2010; Roya et al., 2015).

MATERIALS AND METHODS

Study area

This study was carried out at microbiology laboratory of Nepal Medical College Teaching Hospital, Kathmandu on clinically defined suspected enteric fever cases requesting for blood culture and antibiotic susceptibility testing from March 2012 to September 2012. A total of 1803 blood samples from the febrile ill patients were included in this study.

Microbiology

Blood samples were collected aseptically by venipuncture and inoculated immediately into brain heart infusion broth and incubated at 37°C for 24 h. After incubation, subculture was done on MacConkey agar and Blood agar. Identification of positive culture plates was carried out with the standard microbiological procedure including colony morphology, staining reaction, biochemical characteristics and serotyping using specific antisera (Denka Co. Ltd, Tokyo, Japan). Samples were considered negative for *Salmonella* if no growth was observed until 10 days of incubation (Cheesbrough, 20005).

Antibiotic susceptibility testing

Antimicrobial susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion method with Mueller-Hinton agar using the guidelines and interpretive criteria of the CLSI (CLSI, 2011). Antibiotic discs: ampicillin (10 µg), chloramphenicol (30 µg), cotrimoxazole (1.25/23.75 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), gatifloxacin (5 µg), cefixime (30 µg), cefepime (3 µg), ceftriaxone (30 µg) were tested for all confirmed isolates. *Escherichia coli* ATCC 25922 was used as the quality control strain.

Determination of minimum inhibitory concentration (MIC)

MICs of nalidixic acid, ciprofloxacin, levofloxacin were determined by agar dilution method following CLSI 2011 guideline. *Escherichia coli* ATCC 25922 was used as the quality control strain.

Ethical clearance and consent

Written consent form was obtained from the Institutional Research/ Review Committee (IRC) Nepal Medical College Teaching Hospital at the time of enrollment, prior to commencing the laboratory work and final report was submitted to research and review committee.

Statistical analysis

Statistical analysis was performed using WHONET 5.6 and SPSS 19 software, Student t-test and Chi-square test were used to determine the significant confidence interval (P-value).

Table 1. Month wise distribution of *S*. typhi and *S*. paratyphi A.

Salmonella	Month							Total
	March	April	May	June	July	August	September	
Febrile cases	155	231	302	314	361	242	198	1803
S. typhi	-	3	10	7	1	6	6	33
S. paratyphi A	-	13	24	10	3	6	3	59
Total cases	-	16	34	17	4	12	9	92

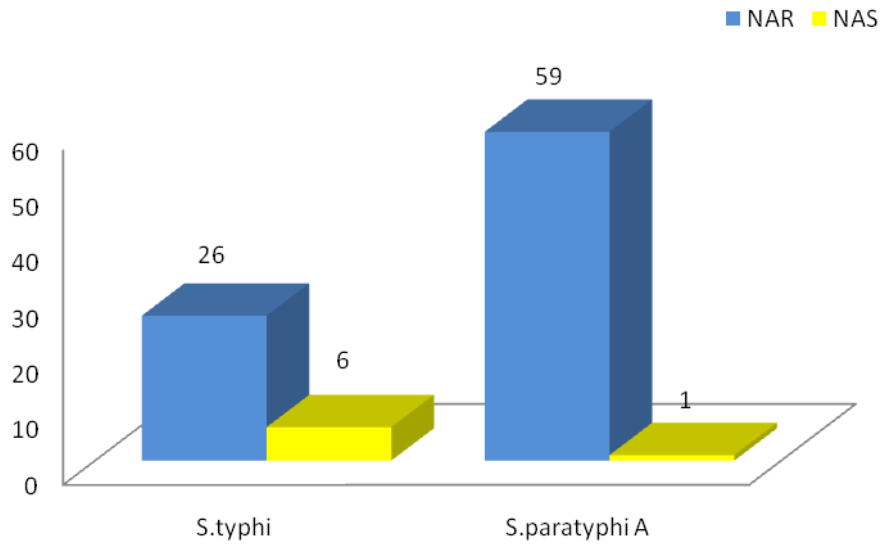

Figure 1. Nalidixic acid resistance pattern in *S*. typhi and *S*. paratyphi A.

RESULTS

A total of 1803 blood culture samples from patients with febrile illness visiting Nepal Medical College Teaching Hospital, Kathmandu, were included in this study. A total of 92 (5.1%) of the blood culture samples were positive for *Salmonella enterica* growth. Serotyping showed that out of 92 isolates, 59 (64.13%) were *S*. paratyphi A and 33 (35.87%) were *S*. typhi. The distribution of these serotypes in age groups varied from 45 days child to 65 years old man with the mean age group of growth 20.59 years. Out of total positive isolates: 62 (67.4%) cases from male, and 30 (32.6%) cases from female (P<0.05). Most of the enteric fever cases were found in the month of May (11.52%) of the total suspected cases. The number of *S*. paratyphi A increases significantly in each month but in August, equal number of *Salmonella* was isolated and in September number of *S*. typhi were greater than the number of *S*. paratyphi A (Table 1).

Antimicrobial susceptibility to quinolone showed that 7 (7.6%) isolates were susceptible and 85 (92.39%) isolates were resistant to nalidixic acid (no zone of inhibition in 30 µg disc). Resistance to nalidixic acid in *S*. typhi and *S*. paratyphi A was 81.81 and 98.30%, respectively (P=0.008) (Figure 1). Overall, nalidixic acid resistance (NAR) was extremely high (92.39%). Among the FQs, newer FQs like gatifloxacin and levofloxacin equally showed highest sensitivity rate (97.82%) followed by ofloxacin (92.39%), ciprofloxacin (88.04%). However, susceptibility to conventional antibiotics (ampicillin, chloramphenicol and co-trimoxazole) was 100% in *S*. paratyphi A and 96.9% in *S*. typhi. Only one isolates (*S*. typhi) (1.08%) was MDR strain. All isolates showed similar sensitivity rate (100%) towards ceftriaxone and cefixime. Similarly, amoxiclillin yielded 100% sensitivity rate towards both *S*. typhi and *S*. paratyphi A, whereas azithromycin displayed 45.5 and 27.12% sensitivity rate towards *S*. typhi and *S*. paratyphi A, respectively. Intermediate strain should be further evaluated by the MIC determination (Table 2).

MIC of quinolone

Of the total isolates, 85 (92.39%) have nalidixic acid MIC of ≥ 32 µg/ml and were classified as resistant, while 7

Table 2. Minimum inhibitory concentration (MICs) of quinolone antimicrobial agents against *Salmonella enterica* isolates by agar dilution method (N= 92).

Serotype	Antibiotics								
	Nalidixic acid			Levofloxacin			Ciprofloxacin		
	S (≤16 µg/ml)	I	R (≥32 µg/ml)	S (≤2 µg/ml)	I (4 µg/ml)	R (≥8 µg/ ml)	S (≤1 µg/ml)	I (2 µg/ml)	R (≥4 µg/ml)
S. enterica typhi(*N*=33)	6	-	27	31	2	-	31	-	2
S. enterca paratyphi A (N=59)	1	-	58	59	-	-	50	9	-

S, Susceptible; I, Intermediate; R, Resistant.

Table 3. Antibiotic susceptibility pattern of *Salmonella enterica* serotypes typhi and paratyphi A by Kirby-Bauer disc diffusion method.

Antiboitic	Serotype typhi (N=33)			Serotype paratyphi A (N=59)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin	32 (96.97)	-	1(3.03)	59 (100)	-	-
Chloramphenicol	32 (96.97)	-	1(3.03)	59 (100)	-	-
Cotrimoxazole	32 (96.97)	-	1(3.03)	59 (100)	-	-
Nalidixic acid	6 (18.18)	-	27(81.82)	1 (1.70)	-	58 (98.3)
Ciprofloxacin	30 (90.91)	1(3.03)	2(6.06)	47 (79.66)	12 (20.34)	-
Ofloxacin	31 (93.94)	-	2(6.06)	54 (91.5)	5 (8.48)	-
Levofloxacin	31 (96.87)	2(9.1)	-	59 (100)	-	-
Gatifloxacin	33 (100)	-	-	59 (100)	-	-
Ceftriaxon	33 (100)	-	-	59 (100)	-	-
Cefixime	33 (100)	-	-	59 (100)	-	-
Azithromycin	15 (45.55)	12(36.36)	6(18.19)	16 (27.12)	29 (49.15)	14 (23.73)
Amoxicillin	33 (100)			59 (100)		

S, Susceptible; I, intermediate; R, resistant.

(7.6%) have MIC of ≤ 8 µg/ml and were classified as susceptible. For ciprofloxacin, 81 (88.04 %) isolates have MIC of ≤ 1 µg/ml and were classified as susceptible, while 9 (9.78%) has MIC of 2 µg/ml, and was classified as intermediate, while 2 (2.17%) has MIC of 4 µg/ml and was classified as resistant. Similarly, for levofloxacin, 90 (97.82%) isolates have MIC of ≤ 2 µg/ml, and classified as susceptible, while 2 (2.44%) have MIC of 4 µg/ml, and classified as intermediate according to CLSI recommendation criteria (Table 3). Based on NA susceptibility, the MIC of ciprofloxacin and levofloxacin for susceptible isolates showed bimodal distribution. MIC of ciprofloxacin ranges from <0.004 to 0.004 µg/ml in NAS isolates, whereas, 0.125 to 1 µg/ml in NAR isolate (Figure 2). Similarly, MIC of levofloxacin ranges from 0.004 to 0.06 µg/ml in NAS isolates, and 0.25 to 2

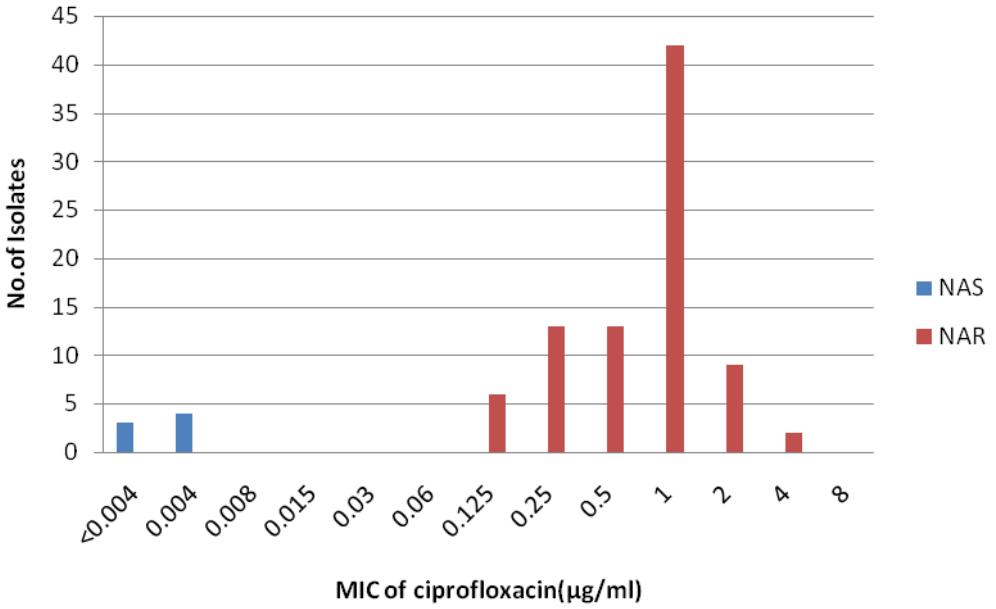

Figure 2. Distribution of MIC of ciprofloxacin between NAR and NAS isolates.

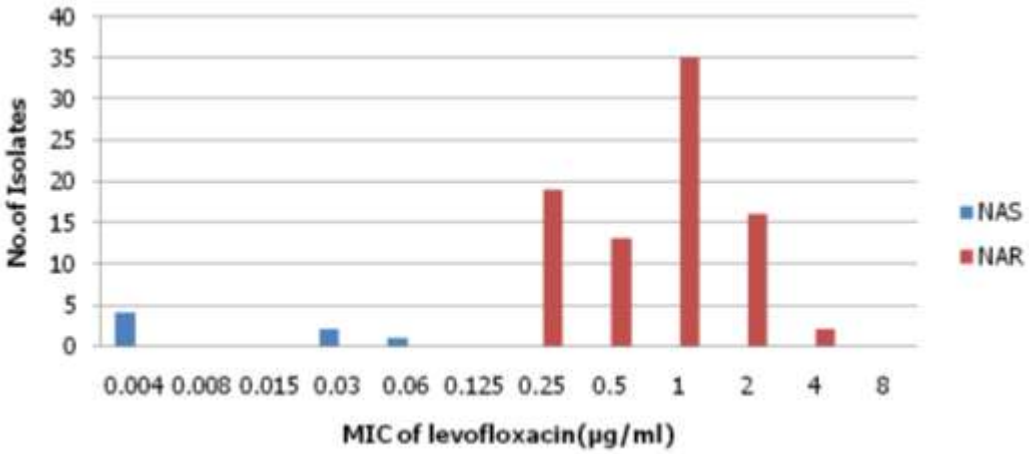

Figure 3. Distribution of MIC of levofloxacin between NAR and NAS isolates.

µg/ml in NAS isolates (Figure 3).

The scatter plots correlate the MICs of levofloxacin and ciprofloxacin with nalidixic acid; demonstrate the simultaneous presence of nalidixic acid resistance and reduced levofloxacin and ciprofloxacin susceptibility. When ciprofloxacin MIC of ≥ 0.125 µg/ml was adopted as a breakpoint, screening for nalidixic acid resistance (MIC ≥32 µg/ml) led to detection of all 85 isolates with reduced ciprofloxacin susceptibility and none of the susceptible isolates. Thus, the sensitivity and specificity of the validity of nalidixic acid screening approach was 100 and 92%, respectively. Similarly, when an levofloxacin MIC of ≥ 0.25 µg/ml was adopted as a breakpoint, screening for nalidixic acid resistance (MIC ≥ 32 µg/ml) led to the detection of all 85 isolates with reduced levofloxacin susceptibility (MIC ≥ 0.25 µg/ml) and none of the susceptible isolates. Thus, the sensitivity and specificity of the approach was 100 and 93%, respectively.

Of the 81 ciprofloxacin susceptible isolates, 74 revealed reduced susceptibility to cirprofloxacin (MIC ≥0.125 µg/ml). Similary, levofloxacin also showed reduced susceptibility (MIC ≥0.25 µg/ml) to 83 levofloxacin susceptible isolates. The mean quinolone MICs in Nalidixic resistant *S.* typhi and *S.* paratyphi A was statistically significant (P<0.001). The NAR *S.* Paratyphi A required increased MICs of the FQs in comparison with

the NAR *S.* typhi. The difference in mean FQs MIC in NAR *S.* typhi and NAR *S.* paratyphi A was statistically significant (P=0.001).

Based on scatter plot analysis, to accommodate a susceptible MIC of ≤1 µg/ml, the zone diameter of 5 µg ciprofloxacin disc for susceptible organism increased to about 24 from 21 mm with corresponding increase in zone diameter for resistant from ≤15 to about 23 mm for resistant MIC of ≥4 µg/ml. Similarly, to accommodate a susceptible MIC of ≤2 µg/ml, the zone diameter of 5 µg levofloxacin disc for susceptible organism increased to about 19 from 17 mm with corresponding increase in zone diameter for resistant from ≤13 to about 17 mm for resistant MIC of ≥8 µg/ml.

DISCUSSION

The shifting of *Salmonella* infection with *S*. Paratyphi A from *S*. typhi, antimicrobial resistance pattern, new approaches to treatment and control strategies, are the rising issues. In this study, the overall growth positivity rate of enteric fever was 5.1%, which is low as compared to the previous studies conducted in Nepal (Malla et al., 2005; Maskey et al., 2008; Shirakawa et al., 2006; Fangtham et al., 2008). Lack of growth in blood culture is common in Nepal mainly because of the use of antibiotics prior to blood collection for culture and moreover misuse of antibiotics even for mild cases of fever is common. More importantly, self-medication is widespread with antibiotics freely available without a prescription (Gupta et al., 2009; Ochiai et al., 2005; Lunn et al., 2010).

Out of 92 *Salmonella* positive cases, 59 (64.13%) were *S*. paratyphi A and 33 (35.87%) were *S*. typhi; indicating higher prevalence of paratyphoid cases than typhoid cases. This finding is higher than the earlier report in Nepal (Shirakawa et al., 2006; Maskey et al., 2008; Pokharel et al., 2009; Acharya et al., 2011). Based on the report from various parts of the world, an estimated one case of paratyphoid fever occurs for every four cases of typhoid fever (Crump et al., 2007). It was reported that in Kathmandu, Nepal, enteric fever caused by *S*. paratyphi A is more prevalent than that caused by *S*. typhi (Shirakawa et al., 2006).

A five year (1994-1998) retrospective analysis at New Delhi in India showed rise in proportion of S. paratyphi A from 6.5 to 44.9%, whereas in Calcutta, isolation rate of S. paratyphi A was 11.1% in 2001 and rocketed to 59% in 2003 (Gupta et al., 2009). Likewise, during 2008–2012 in United States, 2341 enteric fever cases were reported; 80% typhoid and 20% paratyphoid. The proportion caused by paratyphoid A increased from 16 (2008) to 22% (2012) (Date et al., 2016). Nigeria reports the most comprehensive data on *S*. paratyphi from sub Saharan Africa. Nigeria has reported that up to 34 % of enteric fever cases are caused by *S*. Paratyphi A (Akinyemi et al., 2007). Since the past decade, the incidence of *S*. paratyphi A has increased worldwide, moreover in south-central Asia and Southeast Asia countries, it appears to be responsible for up to 50% of blood stream infection (Ochiai et al., 2005; Fangtham et al., 2008). These incidences suggest how rapidly paratyphoid fever increase in various parts of the globe.

Change in host susceptibility, change in virulence of the organism and wide spread use of vaccines and quinolones against S. typhi in the past decade might be major causes of higher proportion of *S*. Paratyphi A in recent years (Gupta et al., 2009; WHO, 2003). In addition to this, in recent years, increased popularity of street food consumption that is also a known risk factor for acquisition of *S.* paratyphi A has been shown (Vollaard et al., 2004). There is a proposed reason that SPA infection is related to higher inocula and ST involves small inocula as food borne transmission is associated with large inocula (Crump et al., 2010).

In this study, rate of NAR, which is a phenotypic marker for reduced susceptibility to fluoroquinolones (Hakanen et al., 1999), was very high (92.39%). *S.* paratyphi A strains showed even higher rate (98.30%) of NAR than *S.* typhi (81.81%). Resistance to NA among *S*. Paratyphi A isolates was recently found to be more common than among *S*. typhi isolates obtained from hospitalized patients in Nepal and India (Acharya et al., 2011; Maskey et al., 2008; Shirakawa et al., 2006).

NAR isolates showed reduced susceptibility to FQs (ciprofloxacin and levofloxacin). Nalidixic acid itself is never used for the treatment of typhoid. However, these isolates are susceptible to FQ in disc sensitivity testing according to current guidelines. The clinical response to treatment with FQs of NAR is significantly worse than with NAS strains. FQs treatment failure has also been reported in patients with NAR *Salmonella* infection (Threlfall et al., 1999). The emergence of NAR *S*. paratyphi A strain is worrying given that ciprofloxacin and ofloxacin are the most commonly used antibiotics for the management of enteric fever in Nepal (Lunn et al., 2010). Apart from these reduced susceptibility in *Salmonella* isolates, complete fluroquinolone resistant *Salmonella* isolates pose a new challenge in the management of enteric fever. In a study carried out in Nepal, all the *S*. Typhi and *S*. Paratyphi A isolates were reported as susceptible until 1998 but during 1999 to 2003, ciprofloxacin resistance increased to 5% in the *S*. Typhi and 13% in *S.* Paratyphi A (Maskey et al., 2008). Another study in Nepal revealed five ciprofloxacin resistant and 7 ofloxacin resistant isolate (Bhatta et al., 2005). A recent study in India showed that Ciprofloxacin resistance was observed in 21% (28/133) of isolates by MIC test (Gopal et al., 2016). Elevated level of reduced susceptibility to fluoroquinolone and even some floroquinolone resistant isolates showed that the treatment of the enteric fever cannot rely on the floroquinolones.

Susceptibility to conventional antibiotics was 100% in *S*. paratyphi A and 96.9% in *S*. typhi showing the decline

of MDR strain and re-emergence of susceptible towards these antibiotics. Other studies from Nepal also found susceptibility towards these conventional antibiotics (Acharya et al., 2011; Shirakawa et al., 2006; Gupta et al., 2009). Based on the observation of re-emergence of susceptibility, conventional first line antimicrobials may play vital role in the management of NAR, and non MDR isolates

In this study, third generation cephalosporin showed 100% sensitive rate towards *S*. paratyphi A. However, extended spectrum beta-lactamase (ESBL) producing *S*. Paratyphi A was isolated in Nepal (Pokharel et al., 2006). Likewise, ESBL producing *S*. paratyphi A was isolated in India (Roy et al., 2015). In addition to this, ESBL producing S. Paratyphi A was isolated from a Japanese traveler to Southeast Asia (Mawatari et al., 2013). Increase incidence of *S*. paratyphi A with decrease susceptibility to fluoroqunilone, fluoroquinolone resistant isolate and ESBL producing isolate limit the treatment of enteric fever. Therefore, early preventive measure like vaccination will be vital in the future to prevent spread in travelers in endemic region as well as native of endemic setting. Currently, the two main vaccines recommended for travelers are the Vi polysaccharide vaccine and the oral Ty21a vaccine. These internationally licensed vaccines are safe and effective against *S*. Typhi. However, there is currently no commercially available vaccine against *S*. paratyphi, which is increasingly reported as a cause of enteric fever (Dave et al., 2015). Recently, French Travelers vaccinated by Vi vaccine against typhoid fever returning from Nepal found *S*. paratyphi A infection (Jean et al., 2016). The fact that current typhoid vaccines have no efficacy against *S.* paratyphi A may interfere to expand typhoid vaccination campaigns for regions with a high incidence of confirmed *S.* typhi disease as this is not likely to solve the problem alone or to make a significant contribution if outbreaks are due to *S.* paratyphi A (Wilde et al., 2007).

In conclusion, this study found 5.1% prevalence, reveals enteric fever is still endemic in an urban setting in Nepal, resulting in significant febrile illness. Moreover, increase incidence of S. paratyphi A with decrease susceptibility to fluoroquinolone demonstrates the need to improve water supply and sanitation system to avoid fecal contamination. This increment of paratyphoid fever will be the new threat for the native as well as non-native. Clinician should be on alert with the treatment with fluroquinolones, as patients with enteric fever due to isolates with decreased fluroquinolone susceptibility, are more likely to have prolonged fever clearance time and higher rates of treatment failure. More comprehensive surveillance of antimicrobial resistance among *S*. paratyphi A strains is warranted in Nepal to determine the extent of geographic expansion of resistant strains from Nepal and to inform treatment options for management of patients. A systematic outbreak investigation to determine source and routes of transmission is recommended.

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Conflict of interest

The authors have not declared any conflict of interest

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