Changing trend of antimicrobial susceptibility pattern of *Salmonella* spp. in different regions of Nepal: A six years surveillance study

**ABSTRACT**

The study was undertaken to evaluate the prevalence of enteric fever, antimicrobial susceptibility pattern, regional and seasonal distribution of *Salmonella* spp. in Nepal. *Salmonella* spp., the causative organism for enteric fever, is a major public health problem; especially in the developing countries and has gradually developed resistance to many antibiotics. Surveillance of antimicrobial resistance is helpful in assessing the burden of disease, trend of antimicrobial resistant pattern and effectiveness of antimicrobials used for treatment of infection. A study was conducted in 11 hospital based laboratories of Nepal from 2005 to 2010. Identification and antimicrobial susceptibility testing of the *Salmonella* isolates was done following standard microbiologic techniques. Isolates received from other laboratories were further confirmed and verified at the National Public Health Laboratory (NPHL). A total of 8342 *Salmonella* isolates were reported during 6 years period of which 5416 (64.92%) were *Salmonella typhi*, 2690 (32.25%) were *Salmonella paratyphi* and 236 (2.83%) were other *Salmonella* spp. The rate of infection was common among 15 to 30 years age group and followed by below 15 years age group in 2007 to 2010. The maximum numbers of isolates were reported during April to June during 2005 to 2007 and in year 2008 and 2010 during July to September, but in 2009 during October to December. 80 to 93% of the *Salmonella* isolates were resistant to Nalidixic Acid (NA). This study also revealed increase in Multidrug Resistant (MDR) *Salmonella* cases (2 to 8%) over the 6 years period. The study shows the prevalence of *Salmonella typhi* over other *Salmonella* spp., and thus, indicates it to be the major causative agent of enteric fever. Although, resistance to most antibiotics is in highly increasing trend, resistance to ceftriaxone was least. Thus, sensitivity pattern of causative organism must be sought before appropriate therapy to prevent further emergence of drug resistance.

**Key words:** *Salmonella*, surveillance, antimicrobial resistance, Nepal.

**INTRODUCTION**

The *Salmonella* spp. infection, ranked as the 13th most common bloodstream infection pathogen during 1997 to 2001 produces diarrheal symptoms and serious invasive infections having high mortality rates, particularly in immune-compromised patients (Stephen et al., 2003). Nepal as well as other South Asian countries falls among the typhoid endemic regions. *Salmonella typhi* is the most common etiological agent, while *Salmonella paratyphi* is responsible for minority of enteric fever cases (Miller et al., 1998; Richens et al., 1996).

From almost every part of Nepal, it has been reported as one of the common cause of febrile illness and is the major reason for seeking health service (Mathura et al., 2005). The multidrug-resistant *Salmonella* spp., was first detected in the mid-1990s in the United States and Europe (Comican et al., 1998; Ribot et al., 2002).
The phenomenon has been attributed to the misuse of antimicrobial drugs favoring the emergence of resistant strains. Antimicrobial-resistant (AMR) organisms lead to increased hospitalizations, health costs, and mortality therefore, becoming an important public health concern associated with serious consequences for the treatment (Kunin et al., 1993; Slark et al., 1989).

Because of adverse health consequences with increasing prevalence of AMR Salmonella, there is an urgent need to emphasize improved sanitation and hygiene, to develop guidelines for prudent usage of antimicrobial agents. Fluoroquinolones were regarded as the drug of choice for treatment of Salmonella infection worldwide, however, due to emerging fluoroquinolone resistant isolates, this study also included many broad spectrum antibiotics as recommended by WHO for treatment of enteric fever.

This study as a part of surveillance program forms the basis to formulate, monitor and identify the prevailing and emerging problem of Multidrug resistant Salmonella spp., which can be contained by effective therapy (Table 1 and 2).

MATERIALS AND METHODS

This study was conducted in 11 major hospital based laboratories of Nepal from 2005 to 2010 (Table 3). Five laboratories based in Kathmandu valley namely: Bir Hospital Laboratory, Patan Hospital Laboratory, Kanti Children’s Hospital (KCH) Laboratory, and Tribhuvan University Teaching Hospital (TUTH) Laboratory including National Public Health Laboratory (National Coordinating laboratory for this study); one in eastern region of Nepal- B. P. Koirala Institute of Health Sciences (BPKIHS) Laboratory, Dharan; four in Western Region of Nepal- Western Regional Hospital Laboratory, Pokhara, Manipal Teaching Hospital Laboratory, Pokhara, United Mission Hospital Laboratory, Palpa, Lumbini Zonal Hospital laboratory, Butwal and Dhulikhel Hospital Laboratory, Kavre. Each participating laboratory had collected Salmonella isolates throughout the year and sent the isolates after confirming the isolates in their laboratory to National Public Health Laboratory (NPHL), the national collaborating (focal point) laboratory.

Study population

This study included all suspected enteric fever patients referred for blood culture by registered physicians.

Collection, transport and enrichment of sample

Adequate amount of blood (5 ml for adults and 3 ml for children) were collected by vein puncture and directly transferred to Brain Heart Infusion Broth (45 ml for adults and 27 ml for children) containing 0.5% bile salts, then, directly transferred to the laboratory as soon as possible. The blood culture vials were then incubated overnight at 37°C.

Processing of sample

The overnight incubated blood sample was streaked onto Mac-Conkey agar and Salmonella-Shigella agar by the quadrant isolation technique. The plates were incubated at 37°C for 24 h. Non-lactose fermenting, smooth, moist pale colonies of 0.5 to 1 mm diameter on Mac-Conkey Agar and colonies of about 0.5 to 1 mm diameter, pale, non-lactose fermenting sometimes with a black centre were suspected to be Salmonella spp. Isolates were identified by Gram’s staining and confirmed by standard 8 tube biochemical tests (Triple sugar Iron test, SIM test, MR-VP test, Urease, Citrate and O/F test).

Inoculum preparation

A sterile loop was used to collect between 4 to 5 well separated colonies from an overnight culture streaked on a nutrient agar plate. The bacteria were suspended into 4 to 5 ml of sterile, normal saline and the turbidity of the suspension was adjusted with sterile saline to obtain a suspension visually similar to that of a 0.5 McFarland standard. The turbidity was read against a standard card with black lines on a white background. A sterile swab was dipped into the suspension and, following removal of excess inoculums by pressing the swab gently against the wall of the tube; bacteria were spread evenly on the Mueller Hinton agar plate.

Antibiotic susceptibility testing

All Salmonella isolates and the control Escherichia coli ATCC 25922 were tested using the KirbyBauer Disc Diffusion method following the “M100-S21- Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement, Vol. 30; No.1” (Clinical and Laboratory Standards Institute (CLSI), 2011 guidelines.

Isolates were tested for susceptibility to the following antibiotics with the respective disc concentrations: Ciprofloxacin (5 mcg), Chloramphenicol (30 mcg), Cotrimoxazole (25 mcg), Ceftriaxone (5 mcg), Nalidixic acid (30 mcg), Tetracycline (30 mcg), Ofloxacin (5 mcg), Gentamicin (10 mcg), Cefixime (30 mcg) and Amoxicillin (10 mcg) (Hi-Media, India). No more than 6 drug-impregnated 6 mm discs were applied to the agar surface.

The discs were manually dispensed using sterile forceps ensuring that discs were no closer than 24 mm from centre to centre. Each disc was pressed gently with sterile forceps to ensure complete contact with the agar and the inoculated...
Table 1. Year wise distribution of Salmonella spp.

<table>
<thead>
<tr>
<th>Year</th>
<th>S. typhi (%)</th>
<th>S. paratyphi (%)</th>
<th>Salmonella spp. (%)</th>
<th>Total isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>570 (82.61)</td>
<td>120 (17.39)</td>
<td>0 (0.00)</td>
<td>690 (8.27)</td>
</tr>
<tr>
<td>2006</td>
<td>1147 (71.20)</td>
<td>448 (27.81)</td>
<td>16 (0.99)</td>
<td>1611 (19.31)</td>
</tr>
<tr>
<td>2007</td>
<td>1118 (73.94)</td>
<td>369 (24.40)</td>
<td>25 (1.65)</td>
<td>1512 (18.13)</td>
</tr>
<tr>
<td>2008</td>
<td>950 (55.98)</td>
<td>615 (36.24)</td>
<td>35 (2.68)</td>
<td>1307 (15.67)</td>
</tr>
<tr>
<td>2009</td>
<td>745 (57.00)</td>
<td>527 (36.24)</td>
<td>35 (2.68)</td>
<td>1307 (15.67)</td>
</tr>
<tr>
<td>2010</td>
<td>886 (58.00)</td>
<td>611 (40.00)</td>
<td>28 (2.00)</td>
<td>1525 (18.28)</td>
</tr>
<tr>
<td>Total</td>
<td>5416 (64.92)</td>
<td>2690 (32.25)</td>
<td>236 (2.83)</td>
<td>8342 (100)</td>
</tr>
</tbody>
</table>

Table 2. Region wise distribution of Salmonella spp.

<table>
<thead>
<tr>
<th>Year</th>
<th>Central region</th>
<th>Western region</th>
<th>Eastern region</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>690</td>
<td>-</td>
<td>-</td>
<td>690</td>
</tr>
<tr>
<td>2006</td>
<td>1574</td>
<td>36</td>
<td>1</td>
<td>1611</td>
</tr>
<tr>
<td>2007</td>
<td>1411</td>
<td>93</td>
<td>8</td>
<td>1512</td>
</tr>
<tr>
<td>2008</td>
<td>1603</td>
<td>84</td>
<td>10</td>
<td>1697</td>
</tr>
<tr>
<td>2009</td>
<td>1267</td>
<td>37</td>
<td>3</td>
<td>1307</td>
</tr>
<tr>
<td>2010</td>
<td>1492</td>
<td>33</td>
<td>-</td>
<td>1525</td>
</tr>
<tr>
<td>Total</td>
<td>8037 (96.34%)</td>
<td>283 (3.39%)</td>
<td>22 (0.26%)</td>
<td>8342</td>
</tr>
</tbody>
</table>

Table 3. Antimicrobial susceptibility patterns of Salmonella during 2005 to 2010.

<table>
<thead>
<tr>
<th>Year</th>
<th>Amp</th>
<th>C</th>
<th>TS</th>
<th>Cip</th>
<th>CRO</th>
<th>NA</th>
<th>T</th>
<th>Of</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>5.00</td>
<td>2.50</td>
<td>2.48</td>
<td>0.00</td>
<td>0.00</td>
<td>80.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>2006</td>
<td>5.87</td>
<td>1.34</td>
<td>2.18</td>
<td>0.96</td>
<td>0.54</td>
<td>73.00</td>
<td>1.15</td>
<td>0.75</td>
</tr>
<tr>
<td>2007</td>
<td>12.3</td>
<td>2.98</td>
<td>5.92</td>
<td>6.48</td>
<td>1.47</td>
<td>64.00</td>
<td>13.00</td>
<td>6.28</td>
</tr>
<tr>
<td>2008</td>
<td>17.01</td>
<td>1.27</td>
<td>3.90</td>
<td>1.75</td>
<td>0.59</td>
<td>83.50</td>
<td>1.37</td>
<td>1.71</td>
</tr>
<tr>
<td>2009</td>
<td>11.84</td>
<td>2.90</td>
<td>6.71</td>
<td>4.33</td>
<td>2.62</td>
<td>78.73</td>
<td>4.41</td>
<td>1.85</td>
</tr>
<tr>
<td>2010</td>
<td>10.33</td>
<td>4.27</td>
<td>6.37</td>
<td>14.04</td>
<td>1.37</td>
<td>93.00</td>
<td>-</td>
<td>3.38</td>
</tr>
</tbody>
</table>

Validation and confirmation of isolates

Validation of the isolates sent from each participating laboratory was done at NPHL following the aforementioned cited procedure. The isolates preliminarily confirmed by colony morphology, gram stain and biochemical test procedures were further validated by serotyping (Denka-Seiken Co., Ltd, Japan).

Briefly, using a wire loop, a small amount of bacteria was picked from a well-isolated colony, transferred onto a glass slide and mixed with a drop of Salmonella poly-O antiserum. The homogenous, slightly milky suspension was tilted back and forth for less than 20 times on the glass slide and the reaction read on a dark surface by naked eye within 1 min from the time of mixing. A drop of sterile physiological saline (0.85% NaCl) instead of the serum was used as a negative control and the test performed simultaneously with the test sample. Isolates that showed agglutination with Poly-O-antisera were further typed with Salmonella antisera O2, O4, O6 and O9 for species differentiation to S. typhi, S. paratyphi A/B/C and Salmonella typhimurium.

RESULTS

A total of 8342 Salmonella isolates were reported during 2005 to 2010 of which 5416 (64.92%) were S. typhi, 2690 (32.25%) S. paratyphi and 236 (2.83%) were other Salmonella spp. (Figure 1). Highest numbers of Salmonella isolates were reported in the year 2008. The trend of predominant of Salmonella spp., was found variable, S. typhi was predominant in 2005 to 2008 and 2010, however, S. paratyphi isolates outnumbered S. typhi in the year 2009. Highest number of Salmonella spp. was recorded in the year 2008.
2008. Highest number of isolates was found to be of *S. typhi* followed by *S. paratyphi* obtained from blood sample each year and only few were obtained from stool, urine and other body fluid.

The rate of Salmonella infection was common among the people below 15 years followed by 15 to 30 years in 2005 and 2006 but the infection rate changed afterwards. The rate of infection was higher among 15 to 30 years age group and followed by below 15 years age group in 2007, 2008, 2009 and 2010.

Figure 2 depicts the frequency of isolation of *Salmonella* spp., throughout the year. For 3 years (2005 to 2007), maximum isolates were reported during April to June. However, the frequency varied in 2008 and 2010 where higher isolates were reported in July to September. In 2009, frequency of isolates was reported higher during October to December. The reason behind this seasonal variation could not be explained. Of the total isolates, 96.34% were isolated in central region, 3.39% in western region and 0.26% in eastern region.
Figure 3. Age wise distribution of *Salmonella* infection.

Figure 4. Seasonal distribution of *Salmonella* isolates.

The antibiotic susceptibility pattern of *Salmonella* spp., during the year 2005 to 2010 is depicted in Figure 3. The table reveals that Salmonella isolates showed increasing resistance rate to various antibiotics. Resistance to Ampicillin (Amp) had increased from 5.00 to 10.33%, Chloramphenicol (C) from 2.50 to 4.27%, Cortimoxazole (TS) from 2.48 to 6.37%, Ciprofloxacin (Cip) from 0.00 to 14.04%, Ceftrixone (CRO) from 0.00 to 1.37%, Nalidixic acid (NA) from 80.00 to 93.00% during 2005 to 2010, Tetracycline (T) from 0.00 to 4.41% during 2005 to 2009, Ofloxacin from 0.75 to 3.38% during 2006 to 2010. Among different antibiotics used, maximum resistance was observed against NA (Figure 4).

On studying the trend of resistance of Salmonella isolates over the 6 years period, multidrug resistance (MDR) isolates (resistance to ampicillin, chloramphenicol and cotrimoxazole) was constant (2%) for years 2005 and 2006 but there was a gradual rise (4%) in 2007, decline (3%) in 2008 which again rose to 7% in 2009 and 8% in 2010. Nalidixic acid-resistant (NAR) isolates decreased from 80% in 2005 to 73% in 2006 and 64% in 2007. However, these strains increased to 84% in 2008, decreased to 79% in 2009 but finally increased to 93% in 2010 which is alarming. Of the total isolates, the highest number of MDR isolates was other *Salmonella* spp., during 2006 to 2010 but in 2005, total MDR isolates belonged to *S. typhi* (Figure 5).

**DISCUSSION**

The present retrospective study describes the isolation and antimicrobial resistance patterns of *Salmonella* spp., for six
years, 2005 to 2010 in Nepal. Antimicrobial drug resistance has become an important public health concern associated with serious consequences for the treatment of infections (Kunin, 1993; Slark, 1989).

In our study, the maximum number of isolates was S. typhi followed by S. paratyphi and other Salmonella spp. Similar results were obtained by Gautam et al. (2002) and Mohanty et al. (2006) during 1999 to 2004, Raveendran et al. (2008) and Kumar et al. (2008), though, the ratio was different. However, Pokharel et al. (2006) reported a lower number of S. typhi as compared to S. paratyphi A.

The highest number of isolates was obtained from blood sample in each year (2005 to 2010) and few isolates were only obtained from stool, urine and other body fluid. This predominance may be due to the large number of blood sample collected in different participating laboratories.

The rate of Salmonella infection was highest among persons below 15 years followed by 15 to 30 years in 2005 and 2006 which was similar to the findings of Pokharel et al. (2009). Sharma et al. (2006) also found salmonella infection commonly among 11 to 20 years group, but the rate of infection was common among 15 to 30 years age group and followed by below 15 years age group in 2007, 2008, 2009 and 2010.

The data generated by this study is contradictory to the study made by Mathura et al. (2005) in which patients having average age of 26.17 years were found to be commonly infected. This variation may be because our study reflects data generated from all regions of Nepal, whereas, Mathura et al. (2005) presented cases visiting only one hospital.

In this study, we found the maximum number of Salmonella isolates during April to June followed by July to September for 3 consecutive years, 2005 to 2007. The finding was similar to Mohanty et al. 2006 and Kumar et al. (2008). Lin et al. (2000) observed the maximum isolates at the end of the dry season in March and April.

According to our findings, in year 2008 and 2010, maximum numbers was obtained during July to September in agreement with the data reported by Madhulika et al. (2004), but in 2009, we observed maximum isolates during October to December with a slight difference. Some other recent studies carried out by Gautam et al. (2002) in India, Ollé-Goig (1993) in Haiti and Velema et al. (1997) in Indonesia have also noted increased isolation particularly at the end of the summer months.

During the dry season, the water level gets progressively lower, becomes more stagnant and potable quality deteriorates as the weather becomes hotter (Lin et al., 2000). Eating ice cream as well as, consumption of food bought from street vendors or cabin during the summer months may also be the reason for ingesting Salmonella resulting in occurrence of maximum isolation of Salmonella during summer. Under these conditions the likelihood of
ingesting Salmonella from contaminated water is high. The non-uniformity in distribution of S. paratyphi cases is in accordance to that noted earlier (Singh et al., 1965).

In our study, the highest numbers of Salmonella spp., were isolated in central region and least in eastern region. The central region consists of 6 sentinel sites, western region 4 and eastern region 1. More sentinel sites in central region may be the reason for isolating maximum number of Salmonella spp., in the central region. The other reason may be difference in eating habit, and the sanitation as well as, hygienic practice of population.

The increasing antibiotic resistivity of Salmonella spp., towards Ampicillin, Chloramphenicol, Cotrimoxazole, Nalidixic acid and Tetracycline was observed during 2005 to 2010 and the maximum resistance was observed against NA. The antibiotic resistivity found in our study is less than in the study done by Gautam et al. (2002) and Raveendran et al. (2008). No resistance was observed in our study for Ceftriaxone as reported by Raveendran et al. (2008), Ciprofloxacin and Tetracycline in 2005 but the resistivity increased during 2006 to 2010.

The percentage of resistivity of Ciprofloxacin was found to be more than Ceftrizone and Tetracycline. Thus, Ceftriaxone may be appropriate in combating the spread of MDR strains. However, it should be used only if the first and second line antibiotics fail to induce a satisfactory response or if the isolate is resistant to Nalidixic acid (Madhulika et al., 2004).

In a study conducted by Raveendran et al. (2008), the resistance pattern of Salmonella spp., varied with time and geographical locations. In our study, MDR isolates increased from 2 to 8% during 2005 to 2010 (Table 4). A significant decline in number of MDR was observed in Karnataka, India by Ciraj et al. (1999), though, the percentage is higher than our finding. The increased trend of MDR percentage was also shown by Gautam et al. (2002) during 1997 to 2001 in India. Eric et al. (2008) found 83.3% MDR S. typhi and Madhulika et al. (2004) found 38.85% MDR Salmonella spp., which is much higher than our finding. The reasons behind this alarming increasing MDR Salmonella spp., may be indiscriminate use of antibiotics and inappropriate prescribing practices by physicians along with intrinsic microbiological plasmid-mediated factors.

As the changing pattern of antibiotic resistivity is observed, it is imperative to get the accurate information on Salmonella isolation and its antibiotic susceptibility pattern to the clinician as soon as possible. A recent study demonstrated that faster reporting with advanced technology (for example, the VITEK system for isolate identification and antibiotic susceptibility testing or automated, computerized laboratory information systems), along with changes in the workflow, could save hospital costs and improve patient care (Barenfanger et al., 1999).

The advanced equipment needed for such rapid reporting is expensive and these methods are also not yet available in most clinical laboratories in developing countries but early diagnosis and appropriate treatment of Salmonella infection is essential to improve the outcome of patients and limit the spread of drug resistant strains.

Our study observed a decline resistivity pattern to NA in the 3 consecutive years, 2005 to 2007, that rose again to 93% till 2010. Our finding is very much similar to Raveendran et al. (2008) and more or less similar to Madhulika et al. (2004). Any isolate that shows NA resistance and susceptibility to ciprofloxacin should be reported intermediately so that clinician could be advised to switch over to another antibiotic (Madhulika et al., 2004) as NA susceptibility has been validated as screening test for reduced susceptibility to ciprofloxacin (Threlfall et al., 2001).

It is notable that the percentage of MDR of other Salmonella spp., has been increasing during 2006 to 2010 and it is predominant than S. typhi and S. paratyphi. This signifies few Salmonella isolate could not be identified to species levels, using the limited set of anti-sera available at NPHL. Most of these unidentified Salmonella species were also MDR. It is advisable that NPHL should continue the surveillance program with complete set of Salmonella anti-sera to identify the emergence of other MDR strains of Salmonella in Nepal.

It is sufficiently clear from the present study that long-term surveillance programmes are essential to identify changes in the epidemiology of enteric fever and to monitor trends in antimicrobial resistance patterns of Salmonella strains isolated which is an important tool to detect the emergence of resistance, to alter treatment strategies and trace the changing susceptibility patterns among microorganisms. The susceptibility pattern might differ from one country to another and also among different regions/locations of the same country. Thus, knowledge of seasonal distribution and antibiotic resistance pattern of Salmonella isolates in particular geographical regions is most helpful in the delineation of appropriate control measures required for prevention of enteric fever (Mohanty et al., 2006).

**CONCLUSION AND RECOMMENDATION**

The study shows that the prevalence of S. typhi, the causative agent of enteric fever is most common among the age group 15 to 30 years. Most of the antibiotics resistance is in increasing order but Ceftrizone showed the slowest rate of resistance. MDR and NA resistant Salmonella spp., are also in increasing order.

Surveillance of the antimicrobial susceptibility profiles of Salmonella spp. should be continued because of the risk for various multidrug-resistant strains detected. Laboratories should develop/ upgrade facility for Salmonella isolation from food samples, complete serotyping of all the Salmonellas spp., develop facilities for molecular studies of drug resistance Salmonella spp., should expand and strengthen other Salmonella testing laboratories.
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REFERENCES


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