Effect of Ciprofloxicine on the growth of *Salmonella* Species

Shweta Verma *, Ahamad Syed Shahab **,
* Dept. of Biotechnology, Sri Satya Sai University of Technology & Medical Sciences Sehore (M.P.)
**Asst. Prof., Sri SatyaSai University of technology & Medical Sciences Sehore (M.P.)

Abstract

Enteric fever, a systemic infection caused by Salmonella enterica Typhi and S. enterica Paratyphi is one of the most common infections in developing countries such as India. Aside from irrational practices of antibiotic use, mutations in chromosomal genes encoding DNA gyrase and Topoisomerase IV and by plasmid mediated quinolone resistant (PMQR) genes are suggested mechanisms for the development of resistance to nalidixic acid and reduced susceptibility to ciprofloxacin. Regardless of high endemicity of enteric fever in India, there is paucity of studies on prevalence and drug-resistance of the pathogen. Therefore, this study aimed to assess the antibiotic susceptibility pattern of Salmonella isolates and determine the minimum inhibitory concentration of ciprofloxacin. A total of 50 blood samples were obtained from patients with suspected enteric fever, attending different samples collected from the hospital during March2020 to February 2021. Blood samples were inoculated immediately into BACTEC culture bottles and further processed for isolation and identification of Salmonella Typhi and S. Paratyphi. Axenic cultures of the isolates were further subjected to antimicrobial susceptibility testing (AST) by using the modified Kirby–Bauer disc diffusion method based on the guidelines by CLSI. The minimum inhibitory concentration (MIC) of ciprofloxacin was determined by agar-dilution method. Out of 50 blood cultures, 39 were positive for Salmonella spp. among which 32 (82.05%) isolates were S. Typhi and 07 (17.9%) isolates were S. Paratyphi A. The MIC value for ciprofloxacin ranged from <125-08 µg/mL in which, respectively, 1,16,6,13,1,1 and 1 of the isolates were susceptible and resistant to ciprofloxacin. Multidrugresistance (MDR) value for Salmonella Typhi and Salmonella Paratyphi were reported 6.83% and 5.12% this study. This study showed high prevalence of quinolone-resistant Salmonella spp., while there was marked re-emergence of susceptibilities to traditional first option drugs. Hence, conventional first-line-drugs and third-generation cephalosporins may find potential usage as the empirical drugs for enteric fever.

Keywords: enteric fever; Salmonella enterica Typhi; S. enterica Paratyphi A; blood culture; PMQR; MIC 1.

Introduction

Enteric fever (typhoid and paratyphoid fever) is a serious bloodstream infection caused by Salmonella enteric Typhi (S. Typhi) and Paratyphi (S. Paratyphi) A, B and C [1]. Enteric fever is transmitted predominantly by the fecal–oral route and manifests with several clinical outcomes including malaise, fever, chills, nausea, abdominal discomfort, Infect [2]. In spite of increased sanitation, personal hygiene, and availability of effective treatment, enteric fever remains as a serious health problem in developing countries. An estimated 11.9–20.6 million cases of typhoid and paratyphoid fever with recorded mortality of 129,000–223,000 are reported annually from developing countries [3]. Moreover, a large proportion of these cases and mortalities is concentrated in South Asia, where it exhibits the seasonal variation, peaking in the rainy reason, from June to August [4]. In case of Indian, the burden of enteric fever is concentrated in the densely populated regions: major city areas[5]. Reduced access to clean drinking water and proper sanitation facilities in these populous regions further deteriorates the quality of life, which leads to the frequent onset of infectious diseases including typhoid and paratyphoid fever. Salmonella has been estimated as the most common single pathogen isolated from blood cultures in India, which is indicative of higher prevalence of enteric fever among Indian population [6]. Antimicrobials such as chloramphenicol, ampicillin, co-trimoxazole and fluoroquinolones are the choice of drugs in the treatment of enteric fever and have proven to be the most effective agents [7]. The case fatality rate is estimated to be 15%–39% without antibiotic therapy, while the fatality rate falls to 2%–7% with the proper treatment course [2]. However, extensive and irrational prescription and use of these drugs have led to the emergence and spread of drug-resistance, often referred as multidrug-resistance (MDR) in the pathogenic strains of Salmonella [6]. MDR strains are responsible for treatment failures, narrowed option of drug regimens, and increased severities and mortalities [8]. The early emergence of drug resistance among Salmonella isolates dates back to late 1980s when the traditional first-line drugs (chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole) became ineffective due to antibiotic-resistance, which compelled

clinicians to rely upon fluoroquinolones, especially ciprofloxacin [9]. Nonetheless, the recent global surge in resistance to fluoroquinolones could lead to a disastrous increase in global infectious diseases [10]. Aside from the inappropriate use of drugs, mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV are described as the reason for quinolones resistance. Subunits of the gyrase and topoisomerase IV are encoded, respectively, by gyrA, gyrB, parC, and parE genes. As the gyrA is the primary target of quinolones, alterations on these determinants help the pathogen to escape the action of antimicrobial agents [11,12]. Moreover, roles of other plasmid-mediated resistant determinants (PMQR) qnr genes and aac (60)-Ib-cr4 also have been described for the resistance to quinolones [13,14]. India has endured several epidemics of enteric fever with varied patterns of drug resistance in the last few decades [15]. To cope with increasing resistance to first-line antibiotics, fluoroquinolones were introduced [17]. However, several findings suggest the increasing burden of nalidixic acid resistant strains with reduced susceptibilities to quinolones, which subsequently has led to the introduction of third generation cephalosporin and azithromycin [18]. Consequently, these newer generations of drugs are also challenged by the MDR strains, as several previous reports have detected such drug-resistant isolates [16,19,20]. Several studies have suggested the re-emergence of antimicrobial susceptibilities of Salmonella spp. to the conventional first-line antibiotics, which can help fight the growing AMR in India [15,17,18,21]. Adequate surveillance and effective infection controls serve as the pillars to curb the burgeoning AMR [22,23]. Despite several previous attempts, there is paucity of reliable data and ample scientific studies on the prevalence and antibiogram of Salmonella spp. This study was conducted to determine the prevalence and antibiotic susceptibility profile of Salmonella spp. isolated from clinical specimens obtained at hospital of Sehore, India. This study further aimed to investigate if there was re-emergence of the susceptibility of the bacterial strains to the conventional drugs, as seen in some previous studies. Infect. Dis. Rep. 2021, 13 390 2.

Material and Methods

Study Design and Sample Population This cross-sectional study was conducted over a period of twelve months (March 2020–February, 2021). A total of 50 non-duplicated blood specimens were collected from the febrile-patients that were suspected to have enteric fever. Suspicion of enteric fever was based on one of the symptoms commonly reported in India. Presence of one of these symptoms was used to suspect typhoid fever that included sustained fever that can be as high as 103 ◦F–104 ◦F; weakness; stomach pain; headache; diarrhea or constipation; cough; and loss of appetite. Since India has a huge burden of typhoid illnesses, any person presenting with fever was suspected of typhoid fever unless it was clinically relatable to other conditions. Suspected patients of all age group and genders who provided written informed consent for their voluntary participation were included in the study. However, patients with incomplete demographic information and those with ongoing or prior antibiotic therapy were excluded from this study. A well-structured, pre-tested questionnaire was administered to each subject to record their demographic information, clinical history and prior antibiotic therapy. Only the recovered isolates of Salmonella were processed for further investigation. Duplication of isolates from the same patient was also avoided.

Sample Collection and Transport Blood specimens were aseptically collected using vein puncture method by an experienced laboratory staff. The volume of blood sample was 8 mL–10 mL for adults and 2 mL–3 mL for pediatric patients. Collected blood samples were immediately transferred to BACTEC culture bottles containing brain heart infusion (BHI) broth (HiMedia, Bengaluru, Karnataka, India) [24].

Laboratory Processing and Identification of the Isolates BACTEC culture bottles inoculated with blood specimens were incubated at 37 ◦C for up to 5 days. Isolates showing growth on BACTEC were further inoculated on conventional culture media such as blood agar (BA), Mac Conkey agar (MA) and xylose lysine deoxycholate agar (XLD). The inoculated culture plates were incubated at 37 ◦C for 18–24 h. The BA plates were used for the observation of non-hemolytic smooth white colonies, MA for nonlactose fermenting colonies and XLD for red colonies with black center [25]. Identification of the isolates was based on colony morphology, Gram staining, and biochemical tests including catalase test, oxidase test, methyl red (MR) test, Voges–Proskauer (VP) test, citrate utilization test, triple sugar iron (TSI) test, sulfide indole motility (SIM) test and urea hydrolysis test (urease test). Antimicrobial Susceptibility Testing (AST) Antimicrobial susceptibilities of the *Salmonella* isolates were tested by using modified Kirby–Bauer disc diffusion in accordance with the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI) [26]. The antibiotic discs used were amoxicillin (AMX, 10 µg), chloramphenicol (C, 30 µg), cotrimoxazole (COT, 25 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), ofloxacin (OF, 5 µg), levofloxacin (LEV, 5 µg), cefixime (CFM, 5 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CTR, 30 µg) and azithromycin (AZM, 15 µg). In this method, broth culture of test organism (comparable to McFarland tube no. 0.5; inoculums density 1.5 × 108 organisms/mL) was uniformly carpeted on the surface of MHA. Then, the antibiotic discs were placed over the lawn culture of the test organism, and the plates were incubated at 37 ◦C for 18 h (or overnight). After Infect. Dis. Rep. 2021, 13 391 incubation, the diameter of zone of inhibition (ZOI) was measured and the results were interpreted as “Resistant” or “Intermediate” or “Susceptible” to that particular antibiotic based on the CLSI guidelines [26]. Salmonella isolates showing resistance to three or more than three

antibiotics of different antibiotic classes were called MDR Salmonella. For instance, *Salmonella* spp. resistant to amoxicillin, chloramphenicol and cotrimoxazole were indicated as MDR Salmonella [27]. Control strains of *Escherichia coli* ATCC (American Type Culture Collection) 25955 and *Staphylococcus aureus* ATCC 25923 were used to ensure the standardization of susceptibility testing. Determination of Minimum Inhibitory Concentrations (MICs) Minimum inhibitory concentration of ciprofloxacin was determined by agar-dilution method [28] based on CLSI guidelines [26]. In this method, MHA plates with various concentration of ciprofloxacin (ranging from 0.0625 µg/mL to 32 µg/mL) were prepared and the test organisms were inoculated on the agar surface. After proper inoculation, the plates were incubated at 37 ◦C for 18–20 h. Following sufficient incubation, the results were interpreted as "sensitive" or "resistant" using breakpoints approved by CLSI [26].

Antibiotic Susceptibility Pattern of *Salmonella* Isolates The highest rate of antibiotic susceptibility was observed towards cefixime (100%) followed by azithromycin (97.5%) and ceftriaxone (95%), whereas the lowest rate of susceptibility was observed towards nalidixic acid (12.5%), followed by ofloxacin (15%) and levofloxacin (20%). All of the isolates were susceptible to all classes of antibiotics used, such as amoxicillin, chloramphenicol, and cotrimoxazole.

Determination of Minimum Inhibitory Concentrations (MIC) of Ciprofloxacin In this study, the MIC values for ciprofloxacin ranged from <125 µg/mL–08µg/mL.

	8ug/ml	4ug/ml	2ug/ml	1ug/ml	0.5ug/ml	0.25ug/ml	0.125ug/ml	<0.125ug/ml
CIPRAFLOXACIN	1	16	6	13	1	0	1	1
GATIFLOXACIN	0	0	0	16	21	1	0	1
LEVOFLOXACIN	0	1	14	8	13	0	0	3

Discussion

Enteric fever is one of the major endemic diseases of low-to-middle-income countries (LMICs) like India. The presence of densely populated urban areas with reduced access to safe drinking water and sanitation, lowered socio-economic status, lack of effective surveillance and poor infection control are some of the driving factors for the high endemicity of the diseases in these countries [29]. Due to the lack of extensive surveillance on the prevalence of enteric fever and unavailability of reliable data on antimicrobial resistance, India is facing several challenges relating to controlling the disease burden and growing drug-resistance [6]. This study was conducted to fulfill a research gap in the country, thereby attempting to estimate the prevalence of enteric fever and the drug-resistance of the pathogens isolated from suspected patients. In addition, we also tried to predict whether there was re-emergence of the susceptibility of enteric pathogens to the conventional first-line drugs. In this study, significant prevalence of enteric fever and drug-resistance profile of Salmonella was observed, which suggests the urgency of intervention measures to counteract the AMR [10]. At the same time, the effectiveness of the first-line antibiotics was well observed, which is suggestive of the possibility of the re-introduction of those antibiotics into the treatment regimens. In this study, less than one-tenth (7.7%) of the total specimens was positive for bacterial growth, among which 3.1% of the specimens was positive for the growth for S. enterica (S. enterica Typhi and S. enterica Paratyphi A). The low growth rate in blood culture can be attributable to the practices of self-medication, which is more common in developing countries [31] including India [32], although we excluded the subjects who had undergone antibiotic therapy for past three months. In this study, the prevalence of S. enterica Typhi was much higher in comparison to S. enterica Paratyphi A Although there is no such well-established reason for serotypic variation in enteric fever, the higher incidence of S. enterica Typhi might be due to the waterborne transmission of S. enterica Typhi, as it usually involves smaller inocula than S. enterica Paratyphi, with the latter being achieved through the food-borne transmission that requires larger inocula [33]. Enteric fever cases occur sporadically throughout the year, peaking in the summer and the rainy season [32,34]. During this season, floods and seepage of water treatment plants and/or sewers can contaminate drinking water and food, thereby resulting in the higher number of typhoid and paratyphoidal cases. There was not any association of gender with the incidence of disease. A slightly different male to female ratio was reported by some previous studies [35,36]. The preponderance of enteric fever among the male population may be explained by their involvement in outdoor activities more frequently, exposing them to the source of infection. The higher concentration of disease burden among younger adults may be attributable to their active social life where the habit of eating out is very likely. In the present study, all the Salmonella isolates were susceptible to chloramphenicol, amoxicillin and cotrimoxazole. Fluroquinolones, especially nalidixic acid are the choice of drugs

against enteric fever in LMICs due to their cost-efficiency, accessibility and availability in oral pills and tablets. However, their long-held utility is now challenged by the burgeoning strains of nalidixicacid-resistant Salmonella (NARS). Genetic factors such as mutations in the genes coding for DNA gyrase (gyrA and gyrB) and topoisomersase IV (parC and pare) are a suggested mechanism for emergence of nalidixic-resistance [37]. Therefore, the role of genetic factors can also be attributable to the high prevalence of NARS in our study; however, dealing with genetic factors was beyond the scope of our study design. A choice of single antibiotic is no longer an effective option in dealing with increasing AMR among Salmonella isolates. As suggested by previous studies, a combination of third-generation cephalosporins and azithromycin can better compensate than when used alone [38]. Our findings also support the effectiveness of these classes of antibiotics, as seen in several previous studies [17,21]. Limitations of the Study This study was conducted in a single hospital for a time period of one year, covering a small population from a limited geographical region. Therefore, the findings of our study did not establish the role of genetic factors in conferring the drug-resistance to the bacterial strains. Therefore, further molecular study consisting of green synthesized nanomaterials in the optical biosensor devices is suggested in future studies to explain the possible mechanisms [39,36].

Conclusions

This study revealed a high rate of resistance among Salmonella isolates to fluoroquinolones, whereas the re-emergence of susceptibility was observed to the conventional drugs including third-generation cephalosporins, which is suggestive of possible re-introduction of such drugs in case management. Our study reported the absence of MDR strains. On the diagnostic side, the detection of drug-resistance by the determination of the MIC can offer better value than using disk-diffusion alone. Proper infection control, the provision of diagnostic facilities, and adherence to the rational practice of antibiotic prescription and use are the recommended measures to cope with the heightened endemicity of enteric fever in the country.

References

1. Banjara, M.R.; Lekhak, B.; Ghimire, P.; Rijal, K.R. Antimicrobial Susceptibility Pattern of Salmonella spp. Isolated from Enteric Fever Patients in Nepal. Infect. Dis. Rep. 2021, 13, 388–400. Crump, J.A.; Mintz, E.D. Global trends in typhoid and paratyphoid Fever. Clin. Infect. Dis. 2010, 50, 241–246.
2. Qian, H.; Cheng, S.; Liu, G.; Tan, Z.; Dong, C.; Bao, J.; Hong, J.; Jin, D.; Bao, C.; Gu, B. Discovery of seven novel mutations of gyrB, parC and parE in Salmonella Typhi and Paratyphi strains from Jiangsu Province of China. Sci. Rep. 2020, 10, 7359.
3. Salerno-Goncalves, R.; Kayastha, D.; Fasano, A.; Levine, M.M.; Sztein, M.B. Crosstalk between leukocytes triggers differential immune responses against Salmonella enterica serovars Typhi and Paratyphi. PLoS Negl. Trop. Dis. 2019, 13, e0007650.
4. Karkey, A.; Arjyal, A.; Anders, K.L.; Boni, M.F.; Dongol, S.; Koirala, S.; My, P.V.; Nga, T.V.; Clements, A.C.; Holt, K.E.; et al. The burden and characteristics of enteric fever at a healthcare facility in a densely populated area of Kathmandu. PLoS ONE 2010, 5, e13988.
5. Environment and Public Health Organization Typhoid: The Neglected Urgent in Nepal. 2019. Available online: http://enpho. org/featured/typhoid-the-neglected-urgent-in-nepal/ (accessed on 27 August 2020).
6. Petersiel, N.; Shrestha, S.; Tamrakar, R.; Koju, R.; Madhup, S.; Shrestha, A.; Bedi, T.; Zmora, N.; Paran, Y.; Schwartz, E.; et al. The epidemiology of typhoid fever in the Dhulikhel area, Nepal: A prospective cohort study. PLoS ONE 2018, 13, e0204479.
7. Ugboko, H.; De, N. Mechanisms of Antibiotic resistance in Salmonella Typhi. Int. J. Curr. Microbiol. App. Sci. 2014, 3, 461–476.
8. Kunwar, D.; Bhatta, S.; Chaudhary, R.; Rijal, K.R. Antibiotic susceptibility pattern of nalidixic acid resistant Salmonella isolates in shree Birendra hospital chhauni. TUJM 2017, 4, 11–14.
9. Rowe, B.; Ward, L.R.; Threlfall, E.J. Multidrug-resistant Salmonella Typhi: A worldwide epidemic. Clin. Infect. Dis. 1997, 24 (Suppl. 1), S106–S109.
10. Ventola, C.L. The antibiotic resistance crisis Part 2: Management strategies and new agents. Pharm. Ther. 2015, 40, 344–352. 11. Hooper, D.C. Bacterial topoisomerases, anti-topoisomerases, and anti-topoisomerase resistance. Clin. Infect. Dis. 1998, 27 (Suppl. 1), S54–S63.
11. Nordmann, P.; Poirel, L. Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. J. Antimicrob. Chemother. 2005, 56, 463–469.
12. Cui, X.; Wang, J.; Yang, C.; Liang, B.; Ma, Q.; Yi, S.; Li, H.; Liu, H.; Li, P.; Wu, Z.; et al. Prevalence and antimicrobial resistance of Shigella flexneri serotype 2 variants in China. Front. Microbiol. 2015, 6, 435.
13. Park, C.H.; Robicsek, A.; Jacoby, G.A.; Sahm, D.; Hooper, D.C. Prevalence in the United States of aac(60)-Ib-cr encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. 2006, 50, 3953–3955.

14. Infect. Dis. Rep. 2021, 13 399 15. Khadka, P.; Thapaliya, J.; Thapa, S. Susceptibility pattern of Salmonella enterica against commonly prescribed antibiotics, to febrile-pediatric cases, in low-income countries. BMC Pediatrics 2021, 21, 38.
15. Pokharel, B.M.; Koirala, J.; Dahal, R.K.; Mishra, S.K.; Khadga, P.K.; Tuladhar, N.R. Multidrug-resistant and extended-spectrum beta-lactamase (ESBL)-producing Salmonella enterica (serotypes Typhi and Paratyphi A) from blood isolates in Nepal: Surveillance of resistance and a search for newer alternatives. Int. J. Infect. Dis. 2006, 10, 434–438.
16. Shrestha, K.L.; Pant, N.D.; Bhandari, R.; Khatri, S.; Shrestha, B.; Lekhak, B. Re-emergence of the susceptibility of the Salmonella spp. isolated from blood samples to conventional first line antibiotics. Antimicrob. Resist. Infect. Control. 2016, 5, 22.
17. Chand, H.J.; Rijal, K.R.; Neupane, B.; Sharma, V.K.; Jha, B. Re-emergence of susceptibility to conventional first line drugs in Salmonella isolates from enteric fever patients in Nepal. J. Infect. Dev. Ctries. 2014, 8, 1483–1487.
18. Shirakawa, T.; Acharya, B.; Kinoshita, S.; Kumagai, S.; Gotoh, A.; Kawabata, M. Decreased susceptibility to fluoroquinolones and gyrA gene mutation in the Salmonella enterica serovar Typhi and Paratyphi A isolated in Katmandu, Nepal, in 2003. Diagn. Microbiol. Infect. Dis. 2006, 54, 299–303.
19. Yu, X.; Zhu, H.; Bo, Y.; Li, Y.; Zhang, Y.; Liu, Y.; Zhang, J.; Jiang, L.; Chen, G.; Zhang, X. Prevalence and antimicrobial resistance of Salmonella enterica subspecies enterica serovar Enteritidis isolated from broiler chickens in Shandong Province, China, 2013–2018. Poult. Sci. 2021, 100, 1016–1023.
20. Khanal, P.R.; Satyal, D.; Bhetwal, A.; Maharjan, A.; Shyakaya, S.; Tandukar, S.; Parajuli, N.P. Renaissance of conventional first-line antibiotics in Salmonella enterica clinical isolates: Assessment of MICs for therapeutic antimicrobials in enteric fever cases from Nepal. Biomed. Res. Int. 2017, 2017, 2868143.
21. Kayastha, K.; Dhungel, B.; Karki, S.; Adhikari, B.; Banjara, M.R.; Rijal, K.R.; Ghimire, P. Extended-spectrum β-lactamase producing Escherichia coli and Klebsiella species in pediatric patients visiting international friendship children's hospital, Kathmandu, Nepal. Infect. Dis. 2020, 13, 1178633720909798.
22. Sah, R.S.P.; Dhungel, B.; Yadav, B.K.; Adhikari, N.; Thapa Shrestha, U.; Lekhak, B.; Banjara, M.R.; Adhikari, B.; Ghimire, P.; Rijal, K.R. Detection of TEM and CTX-M Genes in Escherichia coli isolated from clinical specimens at tertiary care heart hospital, Kathmandu, Nepal. Diseases 2021, 9, 15.
23. Zellweger, R.M.; Basnyat, B.; Shrestha, P.; Prajapati, K.G.; Dongol, S.; Sharma, P.K.; Koirala, S.; Darton, T.C.; Dolecek, C.; Thompson, C.N.; et al. A 23-year retrospective investigation of Salmonella Typhi and Salmonella Paratyphi isolated in a tertiary Kathmandu hospital. PLoS Negl. Trop. Dis. 2017, 11, e0006051.
24. Isenberg, H.D. Clinical Microbiology Procedures Handbook, 2nd ed.; ASM Press: Washington, DC, USA, 2004.
25. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing, 28th ed.; CLSI Supplement; Clinical and Laboratory Standards Institute: Wayne, PA, USA; Volume 38, p. M100.
26. Sivakumar, T.; Avinans, S.N.; Prabhu, D.; Shankar, T.; Vijayabaskar, P. Characterization of multidrug resistant patterns of Salmonella spp. World. J. Med. 2012, 7, 64–67.
27. Andrews, J.M. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. 2001, 48 (Suppl. 1), 5–16.
28. Antillon, M.; Warren, J.L.; Crawford, F.W.; Weinberger, D.M.; Kurum, E.; Pak, G.D.; Marks, F.; Pitzer, V.E. The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. PLoS Negl. Trop. Dis. 2017, 11, e0005376.
29. Prajapati, B.; Rai, G.K.; Rai, S.K.; Upreti, H.C.; Thapa, M.; Singh, G.; Shrestha, R.M. Prevalence of Salmonella Typhi and Paratyphi infection in children: A hospital-based study. Nepal. Med. Coll. J. 2008, 10, 238–241.
30. Parry, C.M.; Hoa, N.T.; Diep, T.S.; Wain, J.; Chinh, N.T.; Vinh, H.; Hien, T.T.; White, N.J.; Farrar, J.J. Value of a single-tube widal test in diagnosis of typhoid fever in Vietnam. J. Clin. Microbiol. 1999, 37, 2882–2886.
31. Malla, S.; Kansakar, P.; Serichantalergs, O.; Rahman, M.; Basnet, S. Epidemiology of typhoid and paratyphoid fever in Kathmandu: Two years study and trends of antimicrobial resistance. J. Nepal. Med. Assoc. 2005, 44, 18–22.
32. Acharya, D.; B, D.; Malla, S.; Dumre, S.P.; Adhikari, N.; Kandel, B.P. Salmonella enterica serovar Paratyphi A: An emerging cause of febrile illness in Nepal. NMCJ 2011, 13, 69–73.
33. Bhattarai, P.M.; Bista, K.P.; Dhakwa, J.R.; Rai, G.K.; Shrestha, R.M.; Thapa, P.B.; Upadhyaya, U.R. A clinical profile of enteric fever at Kanti Children's Hospital. J. Nepal Paediatr. Soc. 2003, 21, 50–53.
34. Ansari, I.; Adhikari, N.; Pandey, R.; Dangal, M.M.; Karanjit, R.; Acharya, A. Enteric fever: Is ciprofloxacin failing? J. Nepal. Paed. Soc. 2002, 20, 6–16.

35. Girard, M.P.; Steele, D.; Chaignat, C.L.; Kieny, M.P. A review of vaccine research and development: Human enteric infections. Vaccine 2006, 24, 2732–2750.
36. Veeraraghavan, B.; Pragasam, A.K.; Bakthavatchalam, Y.D.; Ralph, R. Typhoid fever: Issues in laboratory detection, treatment options & concerns in management in developing countries. Future Sci. OA 2018, 4, FSO312.
37. Ahmadi, S.; Rabiee, N.; Fatahi, Y.; Hooshmand, S.E.; Bagherzadeh, M.; Rabiee, M.; Jajarmi, V.; Dinarvand, R.; Habibzadeh, S.; Saeb, M.R.; et al. Green chemistry and coronavirus. Sustain. Chem. Pharm. 2021, 21, 100415.
38. Rabiee, N.; Bagherzadeh, M.; Ghasemi, A.; Zare, H.; Ahmadi, S.; Fatahi, Y.; Dinarvand, R.; Rabiee, M.; Ramakrishna, S.; Shokouhimehr, M.; et al. Point-of-use rapid detection of SARS-CoV-2: Nanotechnology-enabled solutions for the COVID-19 pandemic. Int. J. Mol. Sci. 2020, 21, 5126.