Research Article

Antibiogram and *Salmonella typhi* detection in suspected patients using advanced PCR technique, serology, and conventional blood culture

Ajaya Jang Kunwar[1,3,4,7]*, Sandeep Thapa [1,3,4], Nilam Thakur [1,3,4,5], Govardhan Joshi [1,3,4], Rajani Shrestha[1], Samikshya Kafle[1,] Sony Shrestha[1,] Kumar Shrestha[2], Kiran Raj Giri[2,] Usha Bogati[2], Junu Budhathoki[2], Suman Kumar Shrestha[3], Santosh Khanal[1,4], Barun Mahat[6,] Dhiraj Maskey[1]

Author's Affiliations

[1]Kathmandu Center for Genomics and Research Laboratory (KCGRL), Lalitpur, Nepal.
[2]St. Xavier's College, Department of Microbiology, Kathmandu, Nepal.
[3]Global Hospital and Education Foundation, Gwarko, Lalitpur, Nepal.
[4]Nova International Diagnostics Pvt. Ltd., Lalitpur, Nepal.
[5]National Academy of Medical Sciences, Bir Hospital, Kathmandu, Nepal.
[6]Nepalese Army Institute of Health Sciences, Kathmandu, Nepal.
[7]Janaki Medical College, Janakpur, Nepal

Correspondence to:
Prof. Dr. Ajaya Jang Kunwar
Department of Anatomy
Janaki Medical College
Email: ajayakunwar@gmail.com

ABSTRACT

Background & Objectives: *Salmonella enterica* serovar *Typhi* is a diverse gram-negative bacillus that causes life-threatening invasive bloodstream infection and remains a public health burden in developing countries. The antimicrobial resistance in *Salmonella typhi* has led to widespread multidrug resistance strains making complication in the treatment and demanding alternative therapies. Various diagnostics test such as blood culture, serological assay, and molecular assay are available. PCR is a newer gold standard method, but blood culture is still considered a definitive diagnosis of typhoid fever.

Materials and Methods: A cross-sectional study was conducted. Venous blood was collected, and a widal test was performed to detect antibodies against O and H antigens in serum. Blood culture, isolation, and identification were performed using the standard techniques. The antibiogram of isolates was performed by the Kirby Bauer method. Amplification of the *hil*A gene through PCR was used for the molecular diagnosis.

Results: Out of 141 suspected typhoid patients, 78 were males, and 63 were females. PCR detected maximum positivity rate (70%) followed by Widal test (43.26%) and blood culture (16.25%). PCR positive cases were highly prevalent in the male in less than 15 years of age group (14.8%), followed

by 15-30 (12.7%) and 30-45 (9.1%). The isolated organism was found to be 100% sensitive to most antibiotics studied, except ofloxacin, which showed 71.4% sensitivity. In contrast, all the isolates were 100% resistant to nalidixic acid. None of the isolates showed multi-drug resistance.

Conclusion: PCR is a sensitive, rapid, and better alternative than conventional methods that can be used in clinical diagnosis for early detection of the *Salmonella typhi.*

Keywords: Antimicrobial susceptibility test (AST)*; hilA;* MDR; PCR; Widal

INTRODUCTION

Enteric fever is a systemic infection caused by the human-adapted pathogens *Salmonella enterica* serotype *Typhi* and *Salmonella enterica* serotype *Paratyphi A, B,* and *C* [1,2]. However, the prevalence of *Salmonella enterica* B and C infection in Nepal are not reported so far. According to WHO, globally, about 16 to 33 million typhoid incidences occur in a year, resulting in 5,00,000 deaths. The incidence of typhoid fever in Nepal is estimated more than 100 per 100,000 cases per year [3].

The study aimed to compare the effectiveness of PCR in detecting Salmonella Typhi to standard diagnostic methods and to assess the isolates' antibiotic susceptibility, with limitations including a small sample size and likely lack of generalizability to larger populations [4,5]. The advent of PCR technology has provided sensitivity and specificity for the diagnosis of typhoid fever [6-10]. The antibiotic sensitivity patterns of *S. Typhi* are changing during recent years, and these need to be continually monitored.

MATERIALS AND METHODS

Data collection was obtained over a course of one year in between 15th July 2014 to 15th July 2015. All the blood samples collected for eligible patients were processed for the Widal test, blood culture, and PCR. Blood samples were obtained aseptically by venipuncture and inoculated into brain heart infusion broth (1:10) with further incubation at 37°C for seven days. Culture bottles were checked for turbidity, gas formation, and other evidence of growth and subculture were performed every subsequent day into MacConkey agar. The isolates were identified by conventional technique, and antibiogram performed using ampicillin (10μg), cefotaxime (30μg), chloramphenicol (30μg), Cotrimoxazole (1.25/23.75 μg), ofloxacin (5μg), nalidixic acid (30μg), azithromycin and tetracycline (30μg) antibiotic discs on Mueller Hinton agar.

DNA was extracted from the blood sample according to standard protocol following the manufacturer's instructions (Nucleo-pore, Genetix). A 20-bp forward primer (5' TAATCGTCCGGTCGTAGTGG-3') and a 20-bp reverse primer (5'-TCTGCAGAAATGGGCGAAAG-3') targeting the *hil*A gene were used in PCR to obtain the 804-bp product. We designed the primer based on the nucleotide accession number NG_034289.1 Salmonella typhimurium activator of invasion gene expression hilA gene. Amplification was carried out in a total volume of 25μL containing 25 pmol each primer, 2.5μM each dNTP, 3 mM $MgCl_2$, 1.5 U Taq DNA polymerase, and PCR buffer. A negative control containing the same reaction mixture except the DNA template was included in every experiment. An initial denaturation at 94^0C for 3 min was followed

by 30 cycles of denaturation at 94^0C for 1 min, annealing at 55^0C for 1 min, and extension at 72^0C for 1 min. Finally, an additional extension was achieved for 10 min at 72^0C. A 10µl aliquot of each PCR product was separated on a 2% agarose gel electrophoresis. PCR was carried out for amplification of hyper invasive locus A (*hil*A) gene.

RESULTS

Out of 141 suspected cases, 21 (15%) samples were culture positive, and 120 (85%) were culture negative. Likewise, 66 (46.8%) samples were found PCR positive, and 75 (53.2%) were PCR negative (Table 1).

Table 1: Comparison of culture and PCR methods for the identification of *Salmonella typhi*

PCR results	No of the cases showing culture results		Total
	Positive	Negative	
Positive	21	45	66 (46.8%)
Negative	0	75	75 (53.2%)
Total	21	120	141 (100.0%)

The 804 base pair *hilA* gene PCR product was seen in agarose gel (Fig 2). All culture-positive samples were PCR positive. PCR positive cases were 37 (47.4%) in males, whereas 29 (46.0%) were female (Table 2).

Table 2. Sex wise distribution of the suspected febrile and typhoid fever cases

Gender	Polymerase chain reaction		Total
	Positive	Negative	
Male	37(47.4%)	41(52.6%)	78
Female	29(46.0%)	34(54.0%)	63
Total	66	75	141

The typhoid cases were found higher in the age group less than 15 years, and the rate was declined in other age groups (Table 3).

Table 3: Age-wise distribution of *Salmonella typhi*

Age groups (in years)	Total cases	PCR		PCR positive %
		Positive	Negative	
<15	38	21	17	21 (14.9%)
16-30	40	18	22	18 (12.7%)
31-45	24	14	10	14 (9.9%)
46-60	22	9	13	9 (6.0%)
61-75	10	3	7	3 (2.1%)
76-90	7	1	6	1 (0.7%)

Antibody titer of 1:80 for O antigen and 1:160 for H antigen were taken as cut-off values to indicate recent typhoid infection. Among, widal tests positive samples, 45 (31.9%) had O antigen, and 52 (36.8%) had H antigen (Table 4).

Table 4. Distribution of Widal test among typhoid fever suspected patients

Antibody type	Cut off titer	Frequency	Percentage (%)
Anti O antibody	≥1:80	45	31.9
Anti H antibody	≥1:160	52	36.8
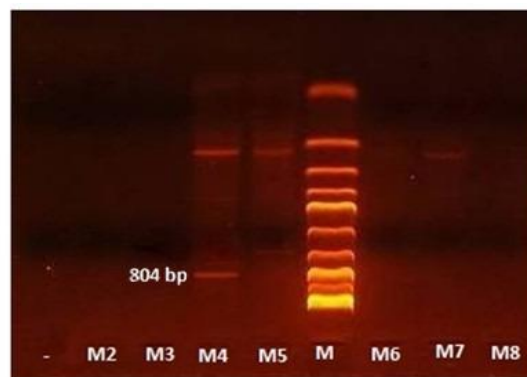
Photograph 4: PCR of positive and negative samples of *Salmonella typhi*. -, Negative control; M, Molecular marker; M2, M3, M5, M6, M7, M8, Negative Samples and M4, Positive sample for *Salmonella typhi*.
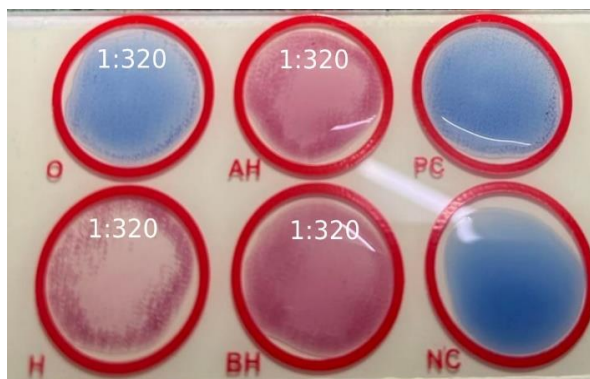
Figure 2. Electrophoresis of *hilA* gene

Kunwar, AJ et al.

Figure 3: WIDAL test (O =1:320, H =1:320, AH =1:320, BH =1:320)

DISCUSSION

Typhoid is a common febrile illness in developing countries [11], including Nepal [12,13]. The disease remains endemic in Kathmandu city because of unsafe drinking water, poor sanitation, low socioeconomic status, unhygienic practices, and cross-contamination of water supply with sewage [12]. Early diagnosis of disease and treatment can be achieved by PCR. Although blood culture was considered a gold standard, but it is a time-consuming technique for isolation and identification of causative agents [14]. Traditional approach like Widal and other serological test have low sensitivity and specificity [15,16].

In our study, among 141 typhoid fever suspected cases, 78 (53.5%) were male and 63 (46.5%) females, but the higher positivity rate was found in males (47.4%) by PCR. Consequently, this study reveals that typhoid fever was more prevalent in the age group of less than 15. Similar results were reported by Pokharel et al. [12]. In contrast, Maskey et al., documented maximum culture positivity in patients between the age group 20-30 years, which covered 51.2% of typhoid fever cases [17]. Similarly, the majority of culture-positive cases were reported in children [10, 18-20]. The higher typhoid positivity in males is likely due to greater environmental exposure and health-seeking behavior differences, while children's prevalence is attributed to weaker immune systems and poorer hygiene practices [21,22].

Blood culture is still the mainstay of the diagnosis of this disease. The overall growth positive rate was 15%, and the rate was relatively higher than the study conducted by others [12,23,24]. In contrast, positive rates of 23.1% and 18.8% were reported by Maskey et al., and Amatya et al., respectively [25,17]. The isolation rate of *Salmonella* species from blood cultures depends on many factors, which includes the volume of blood, the ratio of the volume of blood to the volume of culture broth, the inclusion of anti-complementary substances in the broth, and irrational use of antibiotics [26,27]. Among culture-positive isolates, 100% susceptibility to ampicillin, cefotaxime, chloramphenicol, Cotrimoxazole, and tetracycline, whereas ofloxacin were 71.4% and ciprofloxacin 66.2% sensitive, respectively. Some findings were in agreement with a study conducted at Kanti Children Hospital, Kathmandu, Nepal [28]. However, ceftriaxone was found 100% sensitive in several studies by different authors [13,29,30]. Our study has shown optimum sensitivity to first-line antibiotics, making them a better option for treatment regime for the typhoid fever.

Several reports showed the increasing burden of multi-drug resistant *Salmonella typhi* in various parts of the world like China, India, Indonesia, Pakistan, and Vietnam [31]. In contrast, no multi-drug resistant *Salmonella typhi* (MDRST) was isolated in our study. However, some other studies had reported MDRST from Nepal [13,24,31].

In our study, most of *Salmonella enterica* serovar *Typhi* were isolated in the month of July and August. The typhoid fever outbreak rocketed during the monsoon in Nepal due to possible sewage-mediated water samples contamination during the rainy seasons [12,28,32]. The burden of typhoid fever from June to August is correlated with rainfall which is consistent with our findings [33]. Hiking of typhoid fever during monsoon was quite common in South-Asian countries [34-36]. According to hospital-based surveillance, the number of typhoid fever patients was higher in the summer than in winter [37].

Among 141 blood samples processed for the Widal test, about 31.9% had agglutination reaction with O antigen, whereas 43.2% had agglutination with H antigen. This finding resembles with a study by Gizachew et al.,[16]. Even, positive samples of blood culture for *Salmonella typhi*, 11 (52.3%) had a titer of less than 1:80 in TO, TH which coincides with the study by Hosoglu et al., in which culture-positive (47.6%) samples showed titer greater than 1:80 in TO and TH [38]. Widal tests could be negative in up to 30% of culture-proven cases of typhoid fever, as recommended by WHO (2011). The Widal test remains controversial in diagnosing typhoid fever due to different cut-off values reported in various articles [39,40] and sharing of antigens with other *Salmonella* species and Enterobacteriaceae.

In this study, PCR detected a maximum positivity rate (70%) followed by a Widal test (43.26%) and blood culture (16.25%). PCR indicated positive results of 48.6% typhoid patients that were negative by blood culture and Widal test [41]. This can be evidence that the PCR method is an alternative, rapid, sensitive, and reliable diagnostic technique for the detection of *S. Typhi* in clinically suspected typhoid fever cases as compared to most commonly used conventional methods like blood culture and Widal test. Likewise, Hoque et al., reported 58.2 % and 14.5% positive cases by PCR and blood culture, respectively [7]. Similarly, PCR was more sensitive (84.5%) in a large, well-designed study in Indonesia.[42] Consequently, 82.7% and 26.9% positive were reported in a study from Nepal using PCR and culture, respectively, suggesting that PCR is a much more sensitive and accurate method than others [43]. A small sample size, reliance on a single genetic marker (*hilA* gene) for PCR detection, and no examination of long-term clinical outcomes or the cost-effectiveness of PCR compared to conventional diagnostic procedure are some of the limitations of the study.

CONCLUSION

Typhoid fever is caused by the *Salmonella enterica* serotype *Typhi*, an endemic disease in low-resource countries like Nepal. Conventional techniques are routinely used for diagnosis which have a low accuracy with respect to PCR. Despite limitations like cost and accessibility, PCR method offers rapid and precise pathogen detection. Salmonella typhi isolates were 100% sensitive to certain antibiotics but 100% resistant to nalidixic acid.

ACKNOWLEDGEMENT

REFERENCES

1. Bäumler A, Fang FC. Host specificity of bacterial pathogens. Cold Spring Harb Perspect Med 2013; (12): a010041.
2. Crump JA, Sjölund-Karlsson M, Gordon MA, Parry CM. Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections. Clin Microbiol Rev 2015 ;28(4):901–37.
3. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. Bull World Health Organ 2004;82(5):346–53.
4. Stanaway JD, Atuhebwe PL, Luby SP, Crump JA. Assessing the feasibility of typhoid elimination. Clinical Infectious Diseases 2020;71(2): S179–84.
5. Panzner U, Mogeni OD, Adu-Sarkodie Y, Toy T, Jeon HJ, Pak GD, et al. Detection of Salmonella Typhi nucleic acid by RT-PCR and anti-HlyE, -CdtB, -PilL, and -Vi IgM by ELISA at sites in Ghana, Madagascar and Ethiopia. BMC Infect Dis 2022;22(1):1–15.
6. Frankel G. Detection of Salmonella typhi by PCR. Vol. 32, Journal of clinical microbiology 1994. p. 1415.
7. Haque A, Ahmed N, Peerzada A, Raza A, Bashir S, Abbas G. Utility of PCR in diagnosis of problematic cases of typhoid. Jpn J Infect Dis 2001;54(6):237–9.
8. Hirose K, Itoh KI, Nakajima H, Kurazono T, Yamaguchi M, Moriya K, et al. Selective amplification of tyv (rfbE), prt (rfbS), viaB, and fliC genes by multiplex PCR for identification of Salmonella enterica serovars Typhi and Paratyphi A. J Clin Microbiol 2002;40(2):633–6.
9. Banavandi MJS, Shahhosseiny MH, Shahbazzadeh D, Karimi V, Mirzahoseini H, Mahboudi F, et al. Selective amplification of prt, tyv and invA genes by multiplex PCR for rapid detection of Salmonella typhi. Iran Biomed J 2005;9(3):135–8.
10. Kundu R, Ganguly N, Ghosh TK, Yewale VN, Shah RC, Shah NK. IAP Task Force Report: diagnosis of enteric fever in children. Indian Pediatr 2006;43(10):875–83.
11. Brown JC, Shanahan PM, Jesudason M V, Thomson CJ, Amyes SG. Mutations responsible for reduced susceptibility to 4-quinolones in clinical isolates of multi-resistant Salmonella typhi in India. J Antimicrob Chemother 1996;37(5):891–900.
12. Pokharel P, Rai SK, Karki G, Katuwal A, Vitrakoti R, Shrestha SK. Study of enteric fever and antibiogram of Salmonella isolates at a teaching hospital in Kathmandu Valley. Nepal Med Coll J 2009 ;11(3):176–8.
13. 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. JNMA J Nepal Med Assoc 2005;44(157):18–22.
14. Zhou L, Pollard AJ. A fast and highly sensitive blood culture PCR method for clinical detection of Salmonella enterica serovar Typhi. Ann Clin Microbiol Antimicrob 2010; 9:14.
15. Baker S, Blohmke CJ, Maes M, Johnston PI, Darton TC. The current status of enteric fever diagnostics and implications for disease control. Clinical Infectious Diseases 2020;71(Suppl 2): S64–70.
16. Andualem G, Abebe T, Kebede N, Gebre-Selassie S, Mihret A, Alemayehu H. A comparative study of Widal test with blood culture in the diagnosis of typhoid fever in febrile patients. BMC Res Notes 2014; 7:653.
17. Maskey AP, Basnyat B, Thwaites GE, Campbell JI, Farrar JJ, Zimmerman MD. Emerging trends

in enteric fever in Nepal: 9124 cases confirmed by blood culture 1993-2003. Trans R Soc Trop Med Hyg 2008;102(1):91–5.

18. Walia M, Gaind R, Paul P, Mehta R, Aggarwal P, Kalaivani M. Age-related clinical and microbiological characteristics of enteric fever in India. Trans R Soc Trop Med Hyg 2006;100(10):942–8.

19. Lostroh CP, Bajaj V, Lee CA. The cis requirements for transcriptional activation by HilA, a virulence determinant encoded on SPI-1. Mol Microbiol 2000;37(2):300–15.

20. Bajaj V, Hwang C, Lee CA. hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes. Mol Microbiol 1995 ;18(4):715–27.

21. Amicizia D, Micale RT, Pennati BM, Zangrillo F, Iovine M, Lecini E, et al. Burden of typhoid fever and cholera: similarities and differences. Prevention strategies for European travelers to endemic/epidemic areas. J Prev Med Hyg 2019;60(4): E271.

22. Akullian A, Ng'eno E, Matheson AI, Cosmas L, Macharia D, Fields B, et al. Environmental transmission of typhoid fever in an urban slum. PLoS Negl Trop Dis 2015;9(12): e0004212.

23. Sharma NP, Peacock SJ, Phumratanaprapin W, Day N, White N, Pukrittayakamee S. A hospital-based study of bloodstream infections in febrile patients in Dhulikhel Hospital Kathmandu University Teaching Hospital, Nepal. Southeast Asian J Trop Med Public Health 2006;37(2):351–6.

24. Khanal B, Sharma SK, Bhattacharya SK, Bhattarai NR, Deb M, Kanungo R. Antimicrobial susceptibility patterns of Salmonella enterica serotype typhi in eastern Nepal. J Health Popul Nutr 2007;25(1):82–7.

25. Amatya NM, Shrestha B, Lekhak B. Etiological agents of bacteraemia and antibiotic susceptibility pattern in Kathmandu Model Hospital. JNMA J Nepal Med Assoc 2007;46(167):112–8.

26. Levine MM, Ferreccio C, Black RE, Lagos R, San Martin O, Blackwelder WC. Ty21a live oral typhoid vaccine and prevention of paratyphoid fever caused by Salmonella enterica serovar paratyphi B. Clinical Infectious Diseases 2007 ;45(SUPPL. 1): S24-8.

27. Mohanty SK, Ramana K V. Single and unpaired sera tube widal agglutination test in enteric fever. Vol. 13, Saudi Journal of Gastroenterology India; 2007. p. 213.

28. Prajapati B, Rai GK, Rai SK, Upreti HC, Thapa M, Singh G, et al. Prevalence of Salmonella typhi and paratyphi infection in children: a hospital-based study. Nepal Med Coll J 2008;10(4):238–41.

29. Bhatia JK, Mathur AD, Arora MM. Reemergence of Chloramphenicol Sensitivity in Enteric Fever. Med J Armed Forces India 2007;63(3):212–4.

30. Nair L, Sudarsana J, Pushpa KK. Epidemic of Salmonella enteritica serotype paratyphi A in Calicut-Kerala. Calicut Medical Journal 2004;2(4): e2.

31. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, et al. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ 2008; 86(4):260–8.

32. Acharya D, Bhatta DR, Malla S, Dumre SP, Adhikari N, Kandel BP. Salmonella enterica serovar Paratyphi A: an emerging cause of febrile illness in Nepal. Nepal Med Coll J 2011;13(2):69–73.

33. Karkey A, Aryjal A, Basnyat B, Baker S. Kathmandu, Nepal: still an enteric fever capital of the world. J Infect Dev Ctries 2008;2(6):461–5.

34. Singh R, Singh SP, Ahmad N. A Study of Etiological Pattern in an Epidemic of Acute Febrile Illness during Monsoon in a Tertiary Health Care Institute of Uttarakhand, India. J Clin Diagn Res 2014;8(6):MC01-3.

35. Dewan AM, Corner R, Hashizume M, Ongee ET. Typhoid Fever and its association with environmental factors in the Dhaka Metropolitan Area of Bangladesh: a spatial and time-series approach. PLoS Negl Trop Dis 2013;7(1): e1998.

36. Kumar S, Rizvi M, Berry N. Rising prevalence of enteric fever due to multidrug-resistant Salmonella: an epidemiological study. J Med Microbiol 2008;57(Pt 10):1247–50.

37. Srikantiah P, Vafokulov S, Luby SP, Ishmail T, Earhart K, Khodjaev N, et al. Epidemiology and risk factors for endemic typhoid fever in Uzbekistan. Trop Med Int Health 2007;12(7):838–47.

38. Hosoglu S, Boşnak V, Akalin S, Geyik MF, Ayaz C. Evaluation of false negativity of the Widal test among culture proven typhoid fever cases. J Infect Dev Ctries 2008;2(6):475–8.

39. Olopoenia LA, King AL. Widal agglutination test - 100 years later: still plagued by controversy. Postgrad Med J 2000;76(892):80–4.

40. Wain J, Hosoglu S. The laboratory diagnosis of enteric fever. J Infect Dev Ctries 2008;2(6):421–5.

41. Khan S, Miah RA, Pal S, Khatun S, Fatema N, Roy RR, et al. Comparison of Molecular Detection Method (Nested Polymerase Chain Reaction) with Blood Culture and Paired Widal test for the Rapid Diagnosis of Typhoid Fever. Mymensingh Med J 2017;26(1):117–23.

42. Hatta M, Smits HL. Detection of Salmonella typhi by nested polymerase chain reaction in blood, urine, and stool samples. Am J Trop Med Hyg 2007;76(1):139–43.

43. Ambati SR, Nath G, Das BK. Diagnosis of typhoid fever by polymerase chain reaction. Indian J Pediatr 2007;74(10):909–13.