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Molecular identification of *Salmonella* Paratyphi A by nested PCR from different clinical samples.

Dr Suneel Kumar Ahirwar[1], Dr Chandrabhan Pratap[2], Dr Gopal Nath[3]
Associate professor, Department of Microbiology, Mahatma Gandhi Memorial Medical College, Indore, Madhya Pradesh[1]
Professor, Department of Microbiology, Institute of Medical Science, BHU, Varanasi, Uttar Pradesh[2]
Ex PhD Scholar, Department of Microbiology, Institute of Medical Science, BHU, Varanasi, Uttar Pradesh[3]
Corresponding author
Suneel Kumar Ahirwar
Associate professor, Mahatma Gandhi Memorial Medical College, Indore, Madhya Pradesh
drsk2311@gmail.com

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Abstract

Background and objective *Salmonella* enterica Serovars Paratyphi A, B, and C cause paratyphoid fever, also known as enteric fever, causing symptoms similar to typhoid, such as fevers, chills, and abdominal pain, and can be life-threatening in severe cases. The study utilized nested PCR to efficiently identify small bacterial components, enhancing sensitivity and specificity, saving time, and addressing non-culturable and dead material issues often associated with antibiotic treatment. **Material method** The study, conducted from 2011 to 2013, was conducted in collaboration with the Departments of Medicine and Paediatrics at Sir Sunder Lal Hospital, Institute of Medical Sciences Banaras Hindu University, Uttar Pradesh. DNA extraction from specimens was performed using phenol-chloroform and proteinase K methods. The Van Zwet et al. method was used to extract DNA from 3 to 5 g of stool, mixed with 10% formal saline and ether, and centrifuged at 3,000 rpm for 5 minutes. The nested PCR product was electrophoresed on a 1.5% agarose gel, analyzed using molecular markers, and visualized under ultraviolet illumination. **Result** The study involved 130 participants, including 90 clinically suspected typhoid fever cases and 40 healthy controls, of varying ages and sexes. detection of *Salmonella* paratyphi by nested PCR in blood clots was 25.5%, followed by stool specimens at 42.2% and urine at 42.2%. **Conclusion** The PCR-based method is more sensitive and provides a fast result for detecting *Salmonella* paratyphi A in various specimens compared to blood culture, which takes almost a week.

Key words: *Salmonella* paratyphi A, nested PCR, typhoid fever

Introduction

Salmonella enterica Serovars Paratyphi (S. Paratyphi) A, B, and C are the causes of paratyphoid fever, also known as enteric fever, which includes typhoid fever. *S*. paratyphi serovar A is the most common, but serovar C is less common. Like *S.* typhi, *S*. paratyphi A has adapted to human hosts; it causes symptoms that are similar to those of typhoid, such as fevers, chills, and abdominal pain, and in severe cases, it can be a life-threatening illness. In several developing countries, enteric fever is a serious public health problem. [1,2] Rapid population growth, unplanned urbanisation, inefficient waste management, and inadequate water supply are all attributed for an increase in mortality and morbidity.[3] There were 3.8 million cases of paratyphoid fever globally in 2019, with 23,300 fatalities, a 51.3/100,000 age-standardised incidence rate, and 3.8 million cases (primarily caused by paratyphoid A). [4]. In India, there were 586 cases of typhoid or paratyphoid per 100,000 person-years in 2017, according to the Global Burden of Disease Study. [2]. Serologically based tests include the Widal test and other tests like Typhidot and Tubex that rely on the detection of antibodies in the blood. These assays have low sensitivity and specificity because of the cross-reactivity of antibodies made by conserved Enterobacterales antigens [3,4]. Currently, blood cultures form the cornerstone of enteric fever diagnosis and serve as the "gold standard" against which all other innovative diagnostics are compared. Blood culture sensitivity has been reported to range from 40 to 80%, with sensitivity being higher during the first week of sickness when the bacterial concentration in the blood is many orders of magnitude higher than during succeeding weeks [5]. Blood cultures are less sensitive due to a number of parameters, including the amount of blood that was drawn, the bacteremic level of *S*. paratyphi A, the kind of culture medium, and the length of the incubation period (Wain et al., 2001; Wain et al., 2008) [6]. This has clinical significance because an increasing number of episodes of enteric fever seem to be caused by *S*. paratyphi A [7]. The development of diagnostics to particularly identify cases of *S*. Paratyphi A is inadequate, as efforts are still focused on creating novel tests for *S*. typhi. In this study, nested PCR was utilised to quickly identify very minute quantities of bacterial components, making it possible to identify microorganisms with greater sensitivity and specificity. In addition to saving a large amount of time compared to the usual culture approach and detecting significantly less bacteria, like other organisms, PCR solves the problem of non-

culturable and dead material, which is frequently seen following previous antibiotic treatment.

Material method

The present study was carried out from January 2011 to July 2013 in the department of Microbiology, collaboration with the departments of medicine and paediatrics, at Sir Sunder Lal Hospital, Institute of Medical Sciences Banaras Hindu University, Varanasi, Uttar Pradesh, north India. This study was approved by the ethics committee of the university, and informed written consent was obtained from each of the participants or guardians. 90 clinically suspected cases of typhoid fever were selected from our patient's department and wards of the department of paediatrics and medicine and referred to the department of microbiology. 40 apparently healthy people with no recent history of fever were included as controls. Depending on the patient's age, 5 to 10 ml of blood were taken from each member of the case and control groups. Approximately 5 to 10 g of stool and 40 to 50 ml of urine were collected in a wide-mouthed, clean, sterile universal container. All three types of samples were subjected to culture and nested PCR using primers specific to the *S*. paratyphi *stkG* gene.

Culture of the specimens. About 5 ml of blood were inoculated in brain-heart infusion broth and incubated overnight at 37 °C. After overnight incubation, broth was inoculated on MacConkey agar (MA) and blood agar (BA) and incubated overnight at 37 °C. Negative culture broth was incubated for 7 days, and subcultures were made every alternate day on MA and BA. One loopful of stool sample was directly inoculated on deoxycholate citrate agar (DCA) and MA plates, and 1 to 5 g of stool sample was passed in 10 ml of selenite F enrichment broth. After that, plates and broth were incubated at 37 °C overnight. Subcultures were made from enrichment broth on DCA and MA plates after overnight incubation. About 15 ml of the urine was centrifuged in a falcon tube at 3000 rpm for 5 min. One loopful of deposit from the pellet was inoculated on DCA and MA plates and incubated overnight at 37 °C.

DNA extraction from blood clot, urine, and stool specimens and a nested polymerase chain reaction targeting the stkG primer. Approximately 3 ml of blood allowed to clot, a pellet from centrifugation, and 15 ml of urine from each specimen were subjected to DNA extraction as per the phenol-chloroform and proteinase K methods [8]. 3 to 5 g of stool were added to 10 ml of 10% formal saline (40% [wt/vol] formaldehyde and 0.85% [wt/vol] NaCl) and mixed well to make a suspension. Following this, 3 ml of ether was added and

centrifuged at 3,000 rpm for 5 min. The Van Zwet et al. (9) method was used to harvest DNA from stool specimens (3–5 g) from each study participant.

PCR amplification. The putative fimbrial protein (stkG) gene sequence of S. paratyphi A was used to create oligonucleotide primers (Accession No. CP000026; GI: 56126533).

Primers that target the stkG gene for Salmonella paratyphi

1. Oligonucleotides *stkG* F1 and *stkG* R1 were used for first-round PCR to amplify a 427-bp fragment, which corresponds to nucleotides 96-118 and 522-501, respectively.

stkG F1 5’CGTTTACTGAGGTCACAGGCATC3’

stkG R1 5’CACATTGTTCTCGGAGACCCCA3’

1. Oligonucleotides *stkG* F2 and *stkG* R2 were used for nested PCR to amplify a 299-bp fragment, which corresponds to 138–159 and 366-343, respectively.

stkG F2 5’CAATGGCTTCTGGCGAACTGTC3’

stkG R2 5’TGGAGAAAGATCAGACCACCGAG3’

Master mix for the first round of PCR: 25 µl A master mix was prepared by using 2.5 µl 10x buffer (100 mM Tris-HCl, 1.5 mM MgCl2, and 50 mM KCl) (Genei, Bangalore, India), 2 µl (2.5 mM each) of dNTPs mix (Genei, Bangalore, India), 0.33 µl (1 unit) of Taq DNA polymerase (Genei, Bangalore, India), 10 pmol of each primer, stkG F1 and stkG R1 (SBS Genetech Co., Ltd., Mainland, China), 5 µl of DNA template (100 ng), and remaining deionized water were added to make 25 µl in a composition. Running condition of the thermal cycler and cycle. initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 1 min, with a final extension at 7 min. (35 cycles). Master mix for the nested PCR: all contain ingredients similar to the master mix of the first-round PCR except primers *stkG* F2 and *stkG* R2 and 1 µl of DNA template. Running condition of the thermal cycler and cycle. 94 °C for 5 min, 61 °C for 1 min, and 72 °C for 1 min (35 cycles).

Detection of PCR products The amplified product of nested PCR was electrophoresed on 1.5% agarose gel (Hi-Media, RM 273 Mumbai, India), initially at 100 volts for 5 min and then at constant 80 volts for 60 min with Tris borate EDTA (TBE) buffer. Molecular markers (100-bp DNA ladder, MBI-Fermentas, Germany) were run concurrently. The gel stained with ethidium bromide was visualised under ultraviolet illumination.

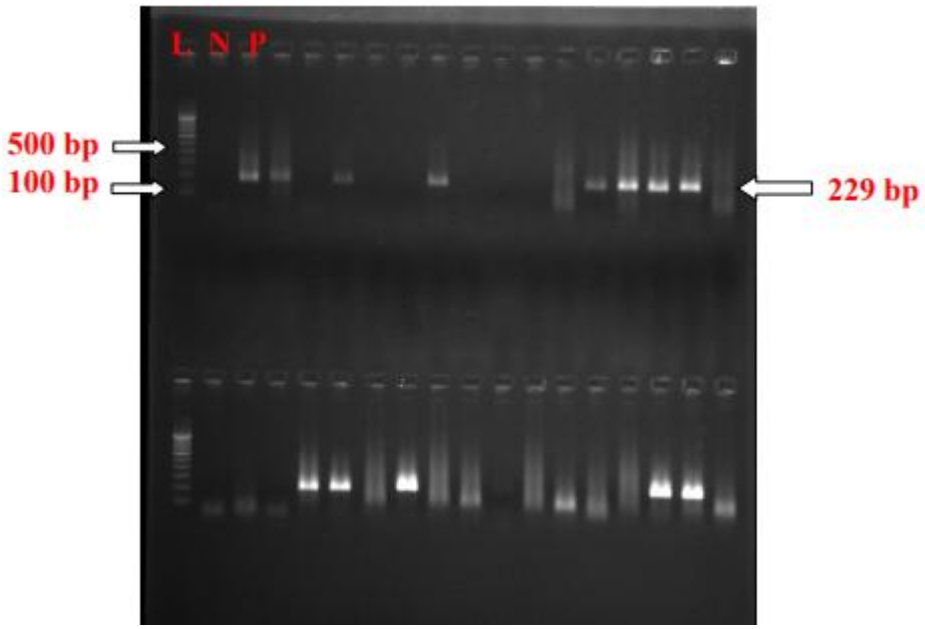
Result

This study was conducted on a total of 130 subjects of different ages and sexes. The study population consisted of 90 clinically suspected cases of typhoid fever and 40 healthy individuals as controls.

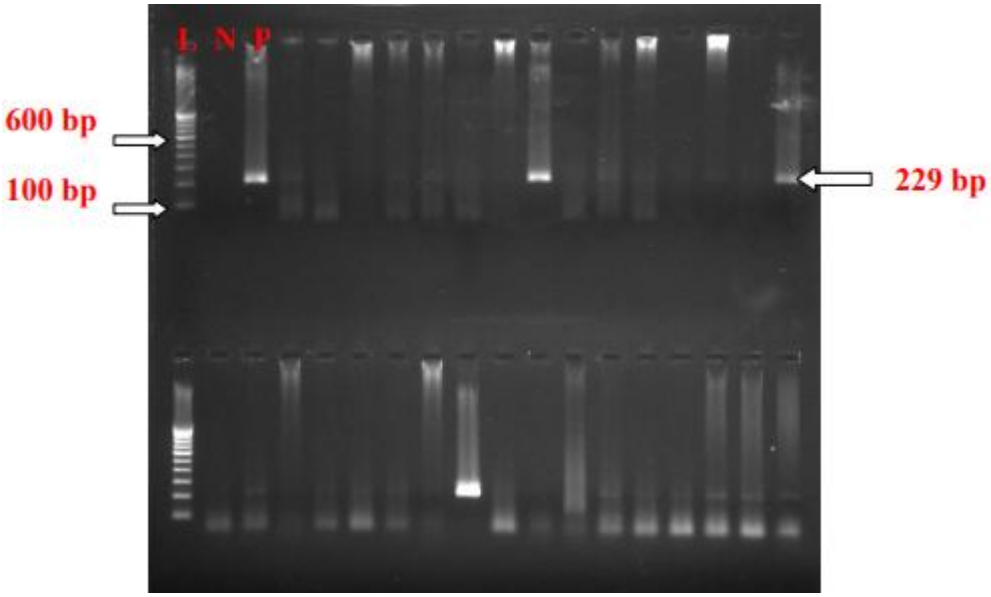
Table 1 detection of salmonella paratyphi by nested PCR

	Blood clot	**Stool**	**Urine**
Conventional culture (group A)	8 (8.8%)	0	0
Nested PCR (group B)	23 (25.5%)	38 (42.2%)	38 (42.2%)

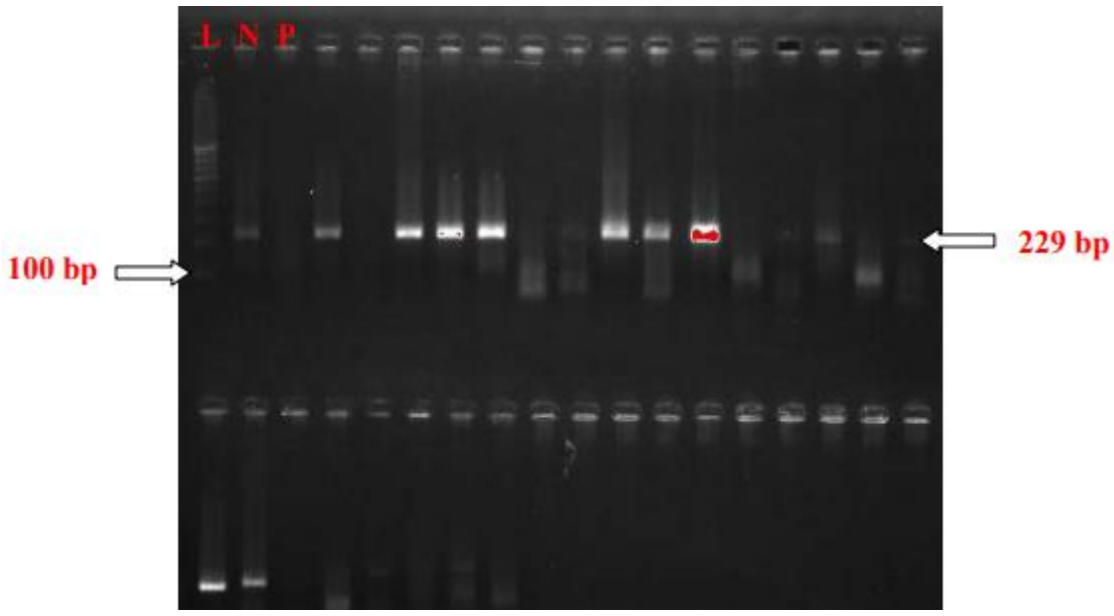
Table 1 showed that the detection of *Salmonella* paratyphi in blood clots was 25.5%, followed by stool specimens at 42.2% and urine at 42.2%.



nPCR for S. Salmonella Paratyphi A stkG (fimbrial) gene (Blood clot)



nPCR for S. Salmonella Paratyphi A stkG (fimbrial) gene (stool specimens)



nPCR for S. Salmonella Paratyphi A stkG (fimbrial) gene (Urine specimens)

Table 2 Mean, median, mode and range of days of illness before presentation of cases, which were positive by various tests.

Test	Range	Mean	Median	Mode
nPCR blood clot for S. Paratyphi	1 - 15	8.6	8	15
nPCR urine for S. Paratyphi	1-18	7.4	7	7
nPCR stool for S. Paratyphi	1 - 25	6.7	6	3

Discussion

Enteric fever is thought to be caused by the human-restricted pathogens *Salmonella* typhi and paratyphi A and B. Globally, there are 20 million cases and 2 lakh fatalities annually. An additional 5 million instances are brought on by paratyphi A annually. *Salmonella* paratyphi, which causes typhoid fever, is becoming more common in the country as a result of contaminated food consumption or a lack of safe drinking water. In our study, 270 specimens, including 90 blood, 90 stool, and 90 urine, were collected from each patient, of which 25.5% from blood clots, 42.2% from urine, and 42.2% from stool were positive for *Salmonella* paratyphi by nested PCR. The isolation rate in cases was found to be 8.8% (8/90). However, none of the 40 afebrile controls yielded positive results for isolation. Amongst them, males (34%) were more affected by Salmonella paratyphi as compared to females (20%). The present finding was similar to the findings of Khosla et al. [10] and Sood and Taneja [11]. This study showed that Salmonella paratyphi affected all age groups, usually 20–30 years (38%), followed by 10–20 years (9.16%), and 1–10 years (7.5%). The positivity of *S*. paratyphi A from all three specimens indicates that the bacteria are also excreted in urine and stool along with circulating in blood. The widal test and blood culture are the two conventionally used diagnostic techniques. Blood culture provides a diagnosis within a week after infection, but it is insensitive because sickness can be caused by as few as 10 bacteria per millilitre [12]. The host immunological response and the intracellular traits of *S*. typhi and paratyphi also have an impact on the blood culture's sensitivity. Molecular techniques, such as polymerase chain reaction (PCR), have revolutionized the field of diagnostic microbiology by providing rapid and highly sensitive methods for detecting pathogens. Nested PCR is a modification of conventional PCR that involves two rounds of amplification, which enhances the specificity and sensitivity of the assay. In the case of *Salmonella* paratyphi, nested PCR can target specific regions of the bacterial DNA and amplify them

to detect the presence of the pathogen in clinical samples. The initial round of PCR amplifies a larger DNA fragment, followed by a second round of amplification using specific primers targeting a smaller region within the first amplicon. This two-step process increases the sensitivity and specificity of the test. The implications of using nested PCR for the molecular diagnosis of *Salmonella* paratyphi are significant. Nested PCR allows for early and accurate detection of the pathogen, even in low concentrations, enabling prompt treatment and better management of the infection. It offers several advantages over traditional culture-based methods, including a shorter turnaround time, improved sensitivity, and the ability to detect nonviable or unculturable bacteria. Additionally, nested PCR can be performed on various types of clinical samples, such as blood, stool, or urine, making it a versatile diagnostic tool. It has also been used in outbreak investigations to identify the source of infection and track the spread of *Salmonella* paratyphi strains. However, it is important to note that while nested PCR is a powerful technique, it requires specialised laboratory equipment and trained personnel to perform the assay correctly. False-positive and false-negative results can occur due to various factors, such as contamination or inhibition of the PCR reaction. In comparison to other approaches, PCR for the identification of typhoidal illnesses is now well established and provides higher sensitivity and specificity. [14, 15, 16]. In order to effectively treat *Salmonella* paratyphi An infection's accurate and early diagnosis is crucial. It would stop the spread of infection in unsanitary settings as well as problems including bleeding and perforation [17]. In order to address the issues of low sensitivity and specificity of conventional diagnostic procedures, PCR in one form or another is being utilised more frequently in normal diagnostic laboratory settings [18].

Conclusion We came to the conclusion that the PCR-based method is more sensitive than blood culture for detecting Salmonella paratyphi A in various specimens. Of course, there's also the added benefit of a fast result—one to two days compared to almost a week for a blood culture.

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