Original Article

NESTED PCR BASED DIAGNOSIS OF *SALMONELLA ENTERICA* SEROVAR PARATYPHI A DIRECTLY FROM BLOOD SAMPLES

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ABSTRACT

Objective: Development of a rapid, reliable PCR - based method for molecular identification of *Salmonella enterica* serovar Paratyphi A directly from blood samples.

Methodology: *S.* Paratyphi A isolates were used for regular PCR targeting specific region of *fliC-a* gene. New primers were designed and conditions were optimized for a nested PCR that could be directly applicable on blood samples. The procedure was tested on 70 blood samples from suspected cases of typhoidal infection and comparison made with blood culture.

Results: Blood culture was able to diagnose only four patients as infected with *S.* Paratyphi A. Regular PCR was unable to detect *S.* Paratyphi A directly from blood where as nested PCR detected *S.* Paratyphi A in blood of thirteen patients.

Conclusions: *S.* Paratyphi A, which is emerging as a major pathogen can be detected with better sensitivity by nested PCR as compared with blood culture.

KEY WORDS: *Salmonella* Paratyphi A, Nested PCR, Paratyphoid Diagnosis.

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INTRODUCTION

Although *S. Typhi* (*Salmonella enterica* serovar Typhi) is still the major cause of typhoidal infection, *S.* Paratyphi A is fast emerging as a major pathogen in developing countries especially in Indian subcontinent.[1] It has a disease presentation highly similar to that of typhoid fever but it seems to follow a distinct route of transmission: whereas typhoid fever is spread predominantly within the household, paratyphoid fever is mainly transmitted outside the patient's home.[2] Clinically, in general, patients with *S.* Paratyphi A enteric fever have more rose spots than patients with typhoid fever.[3] In laboratory, the diagnosis of paratyphoid fever depends on demonstrating the pathogen in blood, bone marrow, stool or urine cultures. However, bacteriological methods are time

consuming and usually require 5-11 days.[4] Additionally, in developing countries like Pakistan sensitivity of blood culture is lowered due to irrational use of antibiotics. The Widal test; a serologic test has a number of limitations including failure to diagnose *S.* Paratyphi A infection.[5,6]

Polymerase chain reaction (PCR), in addition to analysis of foods, has also been successfully applied to the detection and identification of pathogenic organisms in clinical and environmental samples.[7] It has been successfully used for diagnosis of *S.* Typhi and proved superior to conventional methods.[8] A similar approach for diagnosis of *S.* Paratyphi A can be of great help.

Different genes have been targeted to detect *S.* Paratyphi A in water, milk, blood (after spiking with bacteria) and fecal samples but none of them is reported as applicable on patient's blood samples.[9,10] A very specific portion of *fliC-a* gene, present only in *S.* Paratyphi A and not in *S.* Typhi was targeted to identify purified colonies of *S.* Paratyphi A.[11] However, this test involves blood culturing and isolation of bacteria that limits its sensitivity as blood culture is positive in only 50% cases at most.[12] In order to make this technique directly applicable to patient's blood samples with imperative advantages of PCR including better sensitivity and specificity, we designed new internal primers of *fliC-a* gene, developed a nested PCR and optimized it so that it can be successfully applied on blood samples and used for rapid diagnosis of paratyphoid fever.

METHODOLOGY

Five purified *S.* Paratyphi A isolates were taken from NIBGE (National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan) stock cultures. Other related bacteria including *S. Typhi*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Aerobacter* spp., and *Micrococcus* were used as negative controls. The stocks were kept at -20 ºC in TSB (Tryptic soy broth, Merck, Germany) containing 10% dimethyl sulfoxide. For use, these stocks were revived in TSB and cultivated on MacConkey agar overnight to ascertain purity. Isolated colonies were proceeded with to act as controls.

Blood samples from seventy patients, admitted to different hospitals in Faisalabad, Pakistan, having fever for more than three days with enlarged spleen, headache, malaise, abdominal discomfort, and/or agitation, were taken (3ml each) in tubes containing anticoagulant (20mM potassium EDTA), stored at 4ºC and processed for PCR within two days of collection. For blood culture, 2ml of blood was inoculated into a culture bottle containing 16ml of TSB with 0.02% SPS (sodium polyanethanol sulfonate) and incubated at 37ºC for 72 hours. Subculturing was done on MacConkey agar and after overnight incubation, isolated colourless, smooth colonies with 2-3mm diameter were selected for further investigation. Biochemical identification was done by inoculation into Triple Sugar Iron (TSI) medium (Merck, Germany) and results were interpreted according to manufacturer's guidelines. All isolates after biochemical identification were subjected to regular and nested PCR for confirmation.

The genomic DNA from overnight grown culture (3ml each) of purified colonies was extracted by conventional phenol-chloroform method.[13] whereas DNAs from 200µl of each of blood samples were extracted by genomic DNA extraction kit K0512 (Fermentas, USA). The integrity of extracted DNAs was checked by 1% agarose gel electrophoresis. For regular PCR, primers targeting specific region of *fliC-a* gene of *S.* Paratyphi A were used, [11] whereas primers for second round (nested) PCR were designed from the nucleotide sequence database (Genbank [X03393]). Both primer sets were supplied by Sigma (Dorset, United Kingdom) and the sequences are shown in Table-I. The sensitivity of the technique was optimized to be directly applicable on blood samples by procedures as reported for *S. Typhi*.[8] Each 50µL reaction mixture for regular PCR, in addition to 10µL of template, contained 1.5mM $MgCl_2$, 50nmol of each dNTP, 40pM of each primer

and 2U of *Taq* polymerase (Fermentas, Maryland, USA). The thermal cycler (MasterCycler; Eppendorf, Hamburg, Germany) conditions for 30 cycles were as follows; denaturation at 94^{0}C for one minute, annealing at 48^{0}C for one minute and extension at 72^{0}C for 1.5 minutes. For nested PCR, each 50µL reaction mixture, in addition to 10µL template, contained 1.5mM $MgCl_2$, 50nmol of each dNTP, 40pM of each primer and 2U of *Taq* polymerase (Fermentas, USA). The thermal cycler conditions for 30 cycles were as follows; denaturation at 94^{0}C for one minute, annealing at 55 ^{0}C for one minute and extension at 72^{0}C for 1.5 minutes. The amplification products of both regular and nested PCRs were electrophoresed on 2% agarose gel, stained, and photographed by UV transilluminator Eagle Eye (Stratagene, California, USA).

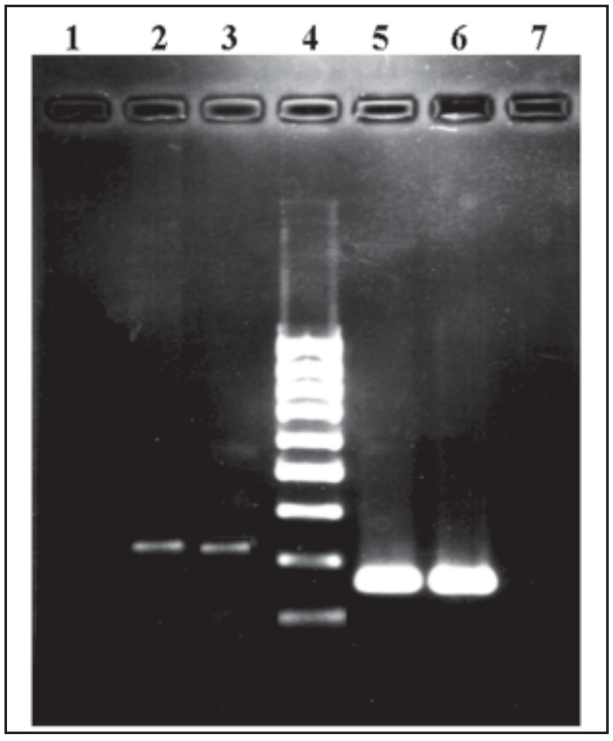

Fig-1: PCR of *S.* Paratyphi A isolates. Lane 4. Molecular weight marker (SM0323S) showing 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 base pairs bands in descending order. Lanes 2 and 3. Regular PCR showing amplified product of 329 bp of *fliC-a* gene. Lanes 5 and 6. Amplified products (259 bp) of *fliC-a* gene by nested PCR. Lanes 1 and 7. Negative controls (without template DNA).

RESULTS

Regular PCR of stock cultures and blood culture isolates of *S.* Paratyphi A amplified a specific region of 329 base pairs (bp) of *fliC-a* gene as previously reported.[11] Optimization of conditions for newly designed primers for nested PCR of these isolates resulted in successful amplification of a 259 bp region of *fliC-a* gene in all *S.* Paratyphi A isolates [Figure-1]. No amplification was obtained using extracted DNAs of the related bacteria *S.* Typhi, *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella aerogenes, Aerobacter* spp., and *Micrococcus* as templates.

From seventy blood samples, blood culture was positive for *S.* Paratyphi A in only four cases. Regular PCR was unable to detect any *Salmonella* directly from blood samples. However, nested PCR detected thirteen blood samples as infected with *S.* Paratyphi A by amplification of *fliC-a* gene (259 bp) [Figure-2].

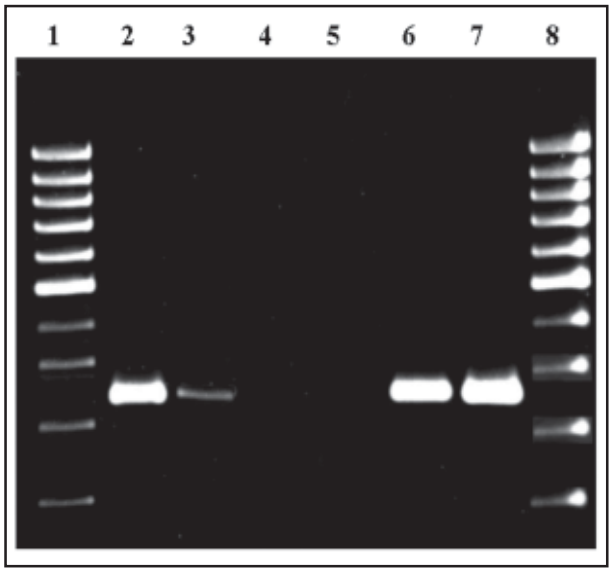

Figure-2: Nested PCR for *S.* Paratyphi A directly from blood samples. Lanes 1 and 8. Molecular weight marker (SM0323S) showing 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 base pairs bands in descending order. Lane 2. Positive control (*S.* Paratyphi A). Lanes 3, 6 and 7. Nested PCR showing amplified product (259 bp) of *fliC-a* gene. Lanes 4 and 5. Patients found negative for *S.* Paratyphi A by nested PCR.

Table-I: Primers used to target *fliC-a* gene of *S.* Paratyphi A.

Primers	*Sequences (5/-3/)*	*Amplicon Size (bp)*	*References*
Regular-1	AATCAACAACAACCTGCAGCG	329	Hirose *et al.*[11]
Regular-2	TAGTGCTTAATGTAGCCGAAGG		
Nested-1	GACCTCGACTCCATCCAGGCTGA	259	This study
Nested-2	TAGTGCTTAATGTAGCCGAAGG		

DISCUSSION

Enteric fever refers to a systemic infection caused by typhoidal *Salmonella*. Classic enteric fever is caused by *S. Typhi* and a similar but relatively less severe syndrome is caused by *S.* Paratyphi A.[14] Up to half of the cases of enteric fever are considered to be due to *S.* Paratyphi A instead of *S. Typhi* that declares *S.* Paratyphi A as a emerging cause of enteric fever in Indian subcontinent including India, Pakistan, China, Nepal, Vietnam and Indonesia.[15] There is no statistically significant difference in patient symptoms and rate of complications between infections due to *S. Typhi* or *S.* Paratyphi A.[16]

For an effective treatment of *S.* Paratyphi A infection, its early and precise diagnosis is important. It would prevent not only complications like haemorrhage and perforation but also the spread of infection in poor hygienic conditions.[17] PCR in one variant or another is being used increasingly in routine diagnostic laboratory settings to overcome the problems of poor sensitivity and specificity of conventional diagnostic methods.[18] *S.* Paratyphi A has already been targeted in water, milk, spiked blood and fecal samples by PCR[9,10] but not directly in clinical blood samples. Regular PCR targeting specific *fliC-a* gene (phase-1 flagellin; H:a) of *S.* Paratyphi A was used with a limited scope of identifying purified bacterial colonies only.[11] We tried to apply this technique of detection of *S.* Paratyphi A directly on blood samples but failed. To overcome this problem, we designed internal primers for the second round (nested) PCR. After optimizing the conditions, nested PCR was applied to seventy clinical blood samples and it successfully identified thirteen patients infected with *S.* Paratyphi A. Specificity of the test was validated as no amplification was found in case of negative controls using DNAs of related bacteria. Blood culture was able to detect only four cases as *S.* Paratyphi A infections. These four cases were also found positive by nested PCR. The poor sensitivity of blood culture is due to multiple factors including host's immune response system[19] that becomes even worse if the patient has already taken some antibiotic drugs which is common practice in this region.

We conclude that this PCR-based detection of *S.* Paratyphi A from blood samples is more sensitive than blood culture. Of course there is additional advantage of a quick result in one to two days as compared with nearly one week required for blood culture.

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