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Original Research Article

Comprism Study between Convential Culturing and Molecular Method for Identification *Salmonella enterica* Serovar *Thphi* in Patients with Typhoid Fever

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Abstract

PCR technique was used for the detection of *S. typhi* and compared to conventional culture results to identify *S. typhi* and determine the relative sensitivity and specificity of these methods. From 254 blood specimens were collected from typhoid fever patients ,207(84.48%) isolated bacteria were obtained and isolated from three groups of patients, group I was positive of PCR and blood culture, group II was positive of blood culture but it was negative of PCR, and group III was positive for intracellular bacteria isolating، methods when it's negative for blood culture and then compared result with typical *Salmonella enterica* serovar *typhi* strain taken from central health laboratory However, 203 (94.4%) isolates were observed positive to nested PCR and 168 (78.1%) was observed positivity by bacteriological and serological methods to identify of isolated bacteria from typhoid patients' blood. When the more specific tests were evaluated in the diagnosis and identify of isolated bacteria that were positive by any of the two tests employed (n = 207), the nested PCR resulted in a relative sensitivity of 98.06% and specificity of 100% when compared with bacteriological and serological methods.

Keywords: *S. typhi*, serology and Blood culture, PCR.

INTRODUCTION

Typhoid fever is an enteric disease and one of the major health problems in the developingcountries, fostered by many interrelated factors, including increased urbanization, inadequatesupplies of clean water, antibiotic resistance, and the variable efficacies of vaccine preparation [1]. *Salmonella* is a member of the family Enterobacteriaceae consist of more than 2500 serovars, and infections caused by *Salmonella* constitute a major public health problem worldwide [2]. These pathogens can affect both human and animals, causing food–borne disease ranging from mild gastroenteritis to life threating systemic infections, such as those caused by *Salmonella enterica* serovar *typhi* known as (*S . typhi*) [3]. Clinical studies demonstrated that *S. typhi* infection stimulates both an intestinal mucosal and systemic humoral and cellular immune response, which are play roles in controlling and clearing *S. typhi* infection [4, 5], by increased levels of circulating proinflammatory and anti-inflammatory cytokines in patients with typhoid and a reduced capacity of whole blood to produce inflammatory cytokines in patients with severe disease [6]. The isolation and identification of *S. typhi* from blood is one of the diagnostic methods of choice for typhoid [7]. But they are labor-intensive and time-consuming which are not suitable for routine testing of large numbers of samples [8]. However, diagnosis of typhoid fever especially in endemic areas where clinical distinguish of typhoid from other febrile illnesses are difficult [9]. Polymerase chain reaction (PCR) is a rapid, sensitive and specific assay for the detection of foodborne pathogens such as *Salmonella* species especially *S. typhi* from different biological samples [5], this technique would be a highly valuable tool for the rapid identification of acute and chronic typhoid infection [10, 11]. For this purpose, rapid detection method (PCR) was used and compared with conventional methods (Serology-Hematology tests (Widal and WBC count tests), and Blood culture) for detection of *S. typhi* from fresh blood specimen of suspected patients with typhoid fever.

MATERIALS AND METHODS

Clinical Specimens

This study included 254 patients represented (124 males and 130 females) with age ranged from 6–60 years, and clinical suspected case of typhoid that came from Al-Kadhimiya Teaching hospital.At period from August to October 2012. Blood samples for

culture, DNA extraction, and serologic analysis were collected from all patients on the same day or within 1–2 days after the first consultation.

Identification of bacterial isolates

Bacterial isolates were identified by inoculation on selective media and biochemical tests, then incubation at 37^{o}C for 24hrs or by serological test and identification rapid system.

Identification of bacteria by selective media

Bacteria were cultured onto selective media include: XLD agar, Bismuth sulfate agar (BSA) and S-S agar media.

Biochemical tests

Production of H_2S and fermenting of sugars

Bacteria were inoculated into tubes containing TSI agar by streaking slant and stabbing butt. After incubation, the colony change on the slant and bottom were identified.

Isolation of the bacterium

Five milliliters of freshly blood was collected and placed in 15mL of Blood culture system (Hi-media) which containing Brain Heart Infusion Broth (BHIB) medium with 0.05% sodium polyanethole sulfonate (SPS), and incubated for 7days at 37°C. One milliliter of this culture was plated on *Salmonella Shigella* (SS) agar and Bismuth sulfate agar (BSA) (Hi-media, India) than incubated for 24hr. at 37°C, and examined for bacterial growth by Gram staining and complete identification by biochemical testing.

Identification of *S. typhi* by Serological test

Identification of *S. typhi* antigens by Slide Test (According to PRO-LAB diagnostics kitprotocol):

A dense suspension of the bacterial cell was prepared in a normal saline and boiled for 10min. or use alcohol dehydrated cells re-suspended in normal saline to Browns tube 2 for identification of somatic antigens. Formalized killed broth culture was prepared for the identification of 'H' antigen. PRO-LAB *Salmonella* anti-sera in a test tube were prepared. Each antiserum must be diluted 1:5 in normal saline before used to prepare of anti-sera dilution. 150µl of normal saline was added to a glass tube and in another tube added an equal volume of diluted anti-sera. An equal volume of previously prepared cell suspension was added to each tube, and incubated in a water bath at 51°C for 2 hours in the case of flagellar antigen identification then observe tubes for agglutination.

Molecular method for diagnosis of *S. typhi*:

DNA extraction:

Extraction of DNA sample from bacterial culture (according to promega protocol).

Extraction of DNA sample from whole blood (according to Promega protocol, 2011)**:**

Determination of the DNA purity by electrophoresis method:

The gel electrophoresis was prepared, sealing ends of gel chamber with tape or appropriate casting system. Put the comb in appropriate of gel tray; 5µl of Ethidium bromide stain was added to cooled gel, the gel for 50% of gel tray was poured and let to solidify for 30min. in a cool place, then the comb was removed and then Casting tray with the gel in it was inserted into electrophoresis running tank, with the wells closest to the negative electrode. Gradually, TAE buffer was added to the tank until buffer just covers the top of the gel, 3µl of sample DNA with7µl of loading dye buffer was prepared previously was added, load samples were added onto gel carefully, the electrophoresed started at 80v for 60 min., and then the band of the DNA sample was seen under UV illumination.

Detection of *S. typhi* by polymerase chain reaction (PCR):

Amplify the DNA samples by PCR:

Twenty five µl from PCR Green master mix was taken and mixed the component by microcentrifuge for 10sec., before starting amplification the DNA samples, Made sure the enterThermo-cycler programed:

For first round of PCR, consisted of 40 cycles of amplification in eppendrof® Thermo cycler were made following below program:

Process	Time	Temperature
Denaturation	1min.	94°C
Annealing	1min.	63°C
Extension	1min.	72°C

For second round of PCR (Nested PCR), consisted of 40 cycles of amplification in eppendroff® Thermo cycler were made following below program.

Statistical Analysis

Diagnostic test calculator Software program was used for statistical analysis the evaluation and comparison between diagnostic tests [12]. Statistical calculator software was used to statistical analysis of significance value (in 0.01 value) of difference mean between two groups was assessed by Independent group's t-test between means.

Process	Time	Temperature
Denaturation	1min.	94°C
Annealing	1min.	63°C
Extension	1min.	72°C

RESULTS AND DISCUSSION

-Identification of *S. typhi* isolates

Morphological Characterization:

After inoculation of sub-culturing, on the MacConkey and blood agar plates either by inoculum of positive blood culture and/or by inoculum of positive isolation intracellular *S. typhi* methods, the appearance of bacterial colonies, were pale or nearly colorless, 1-3mm in diameter, non-lactose fermenting on MacConkey agar, also were whitely to color less colonies, 2-3mm in diameter on blood agar medium without hemolysis of blood [13, 14]. A microscopic examination of this isolated colonies showed that it was gram negative and rod or bacilli in shaped [14], *S. typhi* has different and special characteristics, the appearance of *S. typhi* colonies on a three variety of the most frequently media [15].

Biochemical Tests

The isolates were subjected to biochemical tests for identification. The result showed that the isolates give negative result for urease, indole, citrate utilization and voges- proskaur tests, while resulted positive to methyl red, and glucose fermentation, H2S and no gas production on TSI, the isolates were motile [2]. So according to the biochemical reactions and growth conditions of *S. typhi*, as well as morphological characteristics were identification to that described by [16-19] (Table-1) Morphological and biochemical characteristics of isolate of *S. typhi*.

Table-1: Morphological and biochemical characteristics of isolate of *S. typhi*

No.	Characteristic		Appearance of typical isolated *S. typhi*		
1.	Cell shape		Rod shape		
2.	Gram stain		-		
3.	catalase		+		
4.	oxidase		-		
5.	Motility		+		
6.	TSI	H2S	+		
		Gas	-		
		K/A	+/+		
7.	Indole		-		
8.	Methyl red		+		
9.	Voges- proskaur		-		
10.	Citrate Utilization		-		
11.	Urease		-		
12.	Gelatin liquefaction		+		
13.	Sugars fermentation		Summarized in the table below		
No.	**Sugar**	**Result of ferment**	**No.**	**Sugar**	**Result of ferment**
1.	Glucose	+	8.	Lactose	-
2.	Galactose	+	9.	Sorbitol	+
3.	Fructose	+	10.	Arabinose	-
4.	Sucrose	-	11.	Xylose	+
5.	Mannitol	+	12.	Rhamnose	-
6.	Mannose	+	13.	Rhaffnose	-
7.	Maltose	+			

Where: (+) is indicating that this factor or reaction must be seen in *S. typhi*; (-) indicates thereaction should not appear.
(A): Acidic; (K): Alkaline; (TSI): Triple sugar iron.

Identification of *S. typhi* by serological test and rapid identification system:

- **Identification of *S. typhi* by serological test:**

Agglutination tests were known anti-sera and unknown culture isolate is mixed, and the clumping occurred within few min. So the interpretation of results were, Granular "clumps"observed in the tube are regarded as a positive result for 'O' antigen identification, where as a more floccules appearance observed by using a bright light against a dark background is regarded as a positive result for 'H' antigen identification [20]. The number of bacteria isolates give positive results to serological method was 168, and 69 bacteria isolates were gave negative results.

Identification of *S. typhi* by Nested PCR:

Determining the concentration and purity of DNA sample:

The concentration of DNA samples calculated, the results of DNA purity were high concentration and ranging between 1.2 to 1.61µg/ml. Further the Electrophoresis technique can be consider a good technique to determine the purity of DNA bands appear in agarose gel were photographed.

Comparison and evaluation study between bacteriological and serological methods and nested PCR technique for detection of *S. typhi*:

To investigate the suitability of PCR technique for the detection of S. typhi and compared to conventional culture results to identify S. typhi and determine the relative sensitivity and specificity of these methods. However, 207 isolated bacteria were taken and isolated from three groups of patients, group I was positive of PCR and blood culture, group II was positive of blood culture but it was negative of PCR, and group III was positive for intracellular bacteria isolating methods when it's negative for blood culture and then compared result with typical Salmonella enterica serovar typhi strain taken from central health laboratory (Table-2).

Table-2: Comparison study between detecting *S. typhi* by bacteriological and serological methods and nested PCR from different three groups of patients

No. of isolated bacteria				Type of detection methods	
G I	**GII**	**G III**	**Total**	**(1)**	**(2)**
80	11	73	164	+	+
15	6	18	39	+	-
0	4	0	4	-	+
0	8	22	30	-	-
95	29	114	237	(+)=203/207	(+)=168/207
Control		1		+	+
Probability				1*vs* 2=S	

Where: (1) is nested PCR, (2) is bacteriological and serological methods, (GI) is a positive group for PCR and blood culture, (GII) is a positive group for blood culture but negative to PCR, (GIII) is a positive group for isolate of intracellular bacteria, and S is significant.

However, 203 (94.4%) isolates were observed positive to nested PCR and 168 (78.1%) was observed positivity by bacteriological and serological methods to identify of isolated bacteria from typhoid patients' blood. When the more specific tests were evaluated in the diagnosis and identify of isolated bacteria that were positive by any of the two tests employed (n = 207), the nested PCR resulted in a relative sensitivity of 98.06% and specificity of 100% when compared with bacteriological and serological methods (Table-3).

Table-3: Evaluation of Nested PCR and bacteriological and serological methods to detect of bacterial isolates as *S. typhi* (n= 237)

Test	Types of method	
	Nested PCR	**Bacteriological methods**
Sensitivity (%)	98.06	81.15
Specificity (%)	100.0	100.0
(PV) (+)	1	1
(PV) (-)	0.666667	0.17
(LR) (+)	∞	∞
(LR) (-)	0.02	0.188406

Standardized diagnostic methods to detect the presence of *S. typhi* in blood samples (ISO 6579:2002) are mainly based on bacteriological methods, which in general require up to 3-7 days until significant results are obtained, but sometimes require new modify culture methods to isolate the intracellular bacteria from leukocyte cell [21]. In order to reduce the time demand, alternative techniques like immunological assays [22, 23] and molecular methods [27, 28] have been applied to detect *S. typhi* in several of clinical specimens [24].

Salmonella typhi can be detect more than 98.06% of sensitivity by nested PCR after isolated from blood culture positive or new modify culture methods to isolate the intracellular bacteria from leukocyte cell in the present study, The nested PCR was found highly significant ($P \leq 0.01$) than that of bacteriological and ordinary serological methods for detection of *S. typhi*.

The result in the present study of nested PCR comparison to bacteriological and ordinary serological methods is in agreement with Hatta and Smits, (2007) who reported the sensitivity of nested PCR with blood specimen is 93% with highly significant ($P < 0.01$) when used to diagnose of typhoid fever patients [19]. As mentioned in his thesis, the results of PCR assay

showed sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 100% when Re-evaluation of the PCR assay with DNA of 25 *S. typhi* and 25 non *S. typhi* isolates, he concluded, that *S. typhi* can be detected successfully by PCR.

The reasons for high sensitivity of Nested PCR could be due to high positivity of nested PCR. In the present study might be used and select pair of primers to amplify of the flagellin gene of *S. typhi* identified after isolated from the blood of acute and chronic typhoid patients, the high specificity and sensitivity of primers used to detect of *S. typhi*, when the single round of nested PCR with ST 3 and ST4, amplification products of 343bp were detected from the extracts of *S. typhi* strains but not from the extracts of other organisms [25], and support by [26] who reported it could be used multiplex PCR to differentiated between serogrouping and serotyping of *Salmonella enterica* by specific antigenic targets. The other reason may be due to selected of targeted gene ; because flagellin gene of *S. typhi* has unique nucleotide sequences in the hyper variable region VI of the gene which differ from the other species of *Salmonella* [27], this reasons explains the nested PCR with ST3 and ST 4 were highly sensitive and specific to detect amplification products of *S. typhi* when compared to bacteriological and serological methods to detect *S. typhi* (Table-4) and this explains why (164) of bacteria isolates are really *S. typhi* either by nested PCR or by bacteriological and serological methods, also [28] of bacteria isolates are really *S. typhi* detect by nested PCR, but only four bacterial isolate were *S. typhi* by bacteriological and serological methods but not by PCR, that means false positive of bacteriological and serological methods for detection of *S. typhi* or this bacterial strains are lacking the targeted gene. The negative detection of nested PCR, bacteriological and serological methods to 30 bacterial isolates refers to false positive of blood culture positive and/or infection with other species of *Salmonella* such as *S. paratyphi* A [29].

The whole procedure to identify *S. typhi* DNA in the blood by Nested PCR with electrophoresis taken only six hr. that explained rapidly diagnosis by this method for detection of pathogen; The nested PCR resulted in amplified fragments of bacterial gene that were visible after agarose gel electrophoresis (Figure-1).

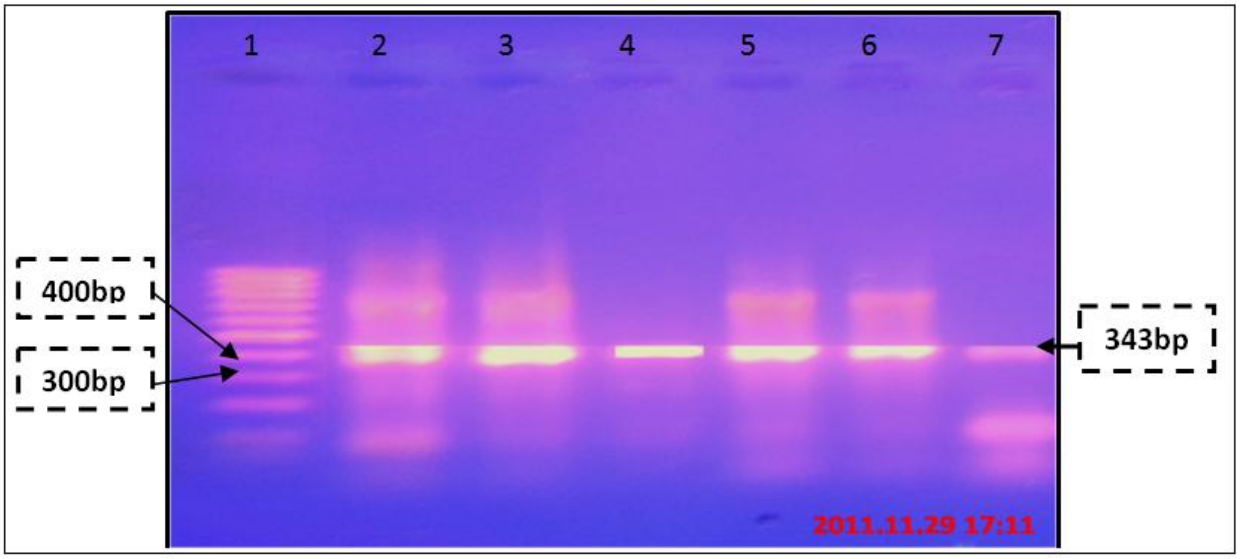

Fig-1: Nested PCR for detection of the flagellin gene of *S. typhi*. Amplification products of 343bp from the single round of PCR were analyzed by electrophoresis through a 1.5% agarose gel for 90 min. at aconstant80 V. Lanes 1 is molecular weight marker (1-kb ladder) (DNA marker), lanes 2 is Positive control DNA isolated from *S. typhi*; lanes 3 to 7, were seen the amplify products with the first round of nested PCR to DNA extracts sample from bacteria isolated

The Positive nested PCR results were in agreement with the finding that have shown promising results due to the rapid, sensitive and specific detection of *S. typhi* [30]. The detection of *Salmonella* serovar *typhi* was first studied by [31] who developed a nested PCR for amplificationof the *fliC* gene of *Salmonella* serovar *typhi* which could detect 5 bacteria/ml and hewas recommend the PCR as a diagnostic tool for detection of this bacteria. Recently, accordingto [32] who reported that a nested multiplex PCR could be used to detect *S. typhi* with asensitivity of10 bacteria/ml. PCR has been widely applicable for early diagnosis of typhoid fever.

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