Evaluation and Comparison of different clinical techniques for detection of local isolate of *Salmonella enterica* serovar *typhi* in patients with typhoid fever

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Abstract:

This study was carried out to evaluate and compare of three clinical techniques which included (Serology-Hematology tests (Widal and WBC count tests), Blood culture, and Polymerase Chain Reaction "PCR") for detection of *Salmonella enterica* serovar *typhi* (*S. typhi*) from fresh blood specimen of suspected patients with typhoid fever.

The positive results for detection of *S. typhi*, that were obtained from these techniques were showed in 192 case (75%), 124 case (48.4%), and 117 case (46.1%) for PCR, Blood culture, and serology-hematology tests respectively from total 254 case of suspected patients infect with typhoid fever were collected in the period of February–June 2011from Al-Kadhimiya Teaching hospital.

When evaluated of this techniques were positive employed (n=232), was show high sensitivity (82.8%) of PCR with higher significant (P < 0.01) while blood culture and Widal test have only (53.45, and 50.43% of sensitivity respectively).

Key word: Typhoid fever, *S. typhi*, Widal test and Blood culture, PCR.

الخـلاصـــــــــــــة

هــذه الدراســةِ تهــدف لتَقيــيم ومقارنــةِ ثــلاث تقنيــاتِ ســريريةِ تَضــمّنتُ (Serology-Hematology tests) (اختبـاري ال Widal and WBCs count tests)، زرع الـدمِّ، والتفاعـل متعـدد السلسـلة "PCR") لتشـخيص بكتريـا *Salmonella enterica* serovar *typhi* (*S. typhi*) مِـنْ عينـات دمّ لمرضى مشكوك اصابتهم بحُمَّى التيفوئيدِ.

وأظهـرت النتـائج الموجبـة للتقنيـات المسـتخدمة ايجابيـة الفحـص فـي ١٩٢ حالـة تمثلـت بنسـبة (٧٥,٦٪)، ١٢٤ حالـة (48.81 ٪)، و١١٧ حالـة (٤٦,١٪) بأسـتخدام تقنيـة PCR ، اختبـار زرع الـدم واختبـاري Widal and WBCs count tests علـى التـوالي مـن مجمـوع ٢٥٤ حالـة مرضـية مشـكوك اصـابتها بحمـى التيفوئيـد والمجموعـة مـن مستشـفى الكاظميـة التعليمـي للفتـرة مـن شـباط - حزيران٢٠١١.

عنـدما قيمـت هـذه الطـرق الـثلاث فـي حالـة النتـائج الموجبـة لهـذه الطرائـق (n= ٢٣٢)، اظهـر اختبـار الحساسـية للطـرق الـثلاث، حساسـية عاليـة (٨٢,٨٪) لتقنيـة PCR مـع فـرق معنـوي عـالي ($P < 0.01$) وحساسـية اقـل لاختبـار زرع الدم واختبار Widal (٥٣,٤٥،٥٠,٤٣ ٪ على التوالي) .

Introduction:

Typhoid fever is an acute systemic disease resulting from infection with *Salmonella enterica* serovar *typhi* (*S*. *typhi*) which is a member of Enterobacteriaceae consist of more than 2500 serovar [(1)], that infect humans and animals to causes spectrum of disease ranging from systemic infection to gastroenteritis, depending on the particular bacterial serovar and the host species infected [(2)]. *S*. *typhi* is gram negative, non-spore forming, rode, and motile bacteria [(3)]. Typhoid can be diagnose by a clinical picture compatible with typhoid and significant titer of agglutination antibodies in the blood against H and/or O antigen of *S. typhi* [(4)].

The isolation and identification of *S. typhi* from blood is one of the diagnostic methods of choice for typhoid [(5)]. But they are labor-intensive and time-consuming which are not suitable for routine testing of large numbers of samples [(6)]. However, diagnosis of typhoid fever especially in endemic areas where clinical distinguish of typhoid from other febrile illnesses are difficult [(7)]. 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 [(8)], this technique would be a highly valuable tool for the rapid identification of acute and chronic typhoid infection [(9; 10)].

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.

Material and Methods:

Selection of the clinical cases:

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.

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.

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 and rapid identify system (EPI 25).

Serologic analysis:

The Widal test with O and H antigens (Linear, Spanish) was performed and interpreted according to routine laboratory procedures. A titer 1:160 was considered positive result.

Hematological investigations:

Total leucocytes count and differential leucocytes count were performed and interpreted according to routine laboratory procedures.

Isolation of genomic DNA:

Genomic DNA from patients blood and cultured bacterial were extracted according to the protocol's instructions of Wizard kit for isolate and identifies of DNA (Promega, USA), the purity of the extracted DNA was checked either by measurement A260 and A280 or by electrophoresis.

Molecularanalysis:

PCR primers: Two pairs of oligonucleotide primers according to [11], were used in this study synthesized by Alpha DNA synthesizer which includes: forward primer (ST1): 5'-ACTGCTAAAACCACTACT-3', reverse primer (ST2): 5'-TTAACGC AGTA AAGAGAG-3' which were used in the first round of PCR to amplify a 458-bp fragment correspond to nucleotides 1072 to 1089 and 1513 to 1530, respectively. Second oligonucleotides primer includes forward primer (ST3): 5'-AGATGGTACTGGCGTTGCTC-3' and reverse primer (ST4): 5'-TGGAGAC TTCGGTCGCG TAG-3' which were used in the nested PCR on the amplified products from the first PCR to amplify a 343-bp fragment correspond to nucleotides 1092 to 1111 and 1416 to 1435, respectively.

PCR condition:

500µl of reaction mixture supplied from (Promega, USA) was prepared for 20 samples (25µl for each one) contain100µl of 1× PCR buffer, 20µl (25pmol) for each primer ST1 and ST2, 10µl (200µM) for each of DNTP (deoxyribonucleic triphosphate), 65µl (0.625U) of GoTaq® DNA polymerase, 40µl (2µg) of DNA template, and water to a final volume of 500µl. The first round amplification was carried out in a thermo cycler (eppendroff®, USA) under the following conditions: 40 cycles represented by 1min of denaturation at 94°C, 1min of annealing at 63°C, and 1min of extension at 72°C. The nested PCR master mix and amplify condition was the same as that accomplished of the first round of PCR. To separate amplified products, 5µl of solution product and molecular size markers (1kb DNA ladder) supplied from Promega(USA) were electrophoresed on a 1.5%

agarose gel in TBE (Tris-borate-EDTA) buffer at 80V for 90min., and then the gel were stained with Ethidium bromide (EB), and the band were visualized under UV illumination.

Statistical analysis:

The statistical analysis was calculated by *t* test was applied to determine the significance differences between the clinical techniques.

Results:

Three diagnostic methods represented in PCR, blood culture and Widal/WBCs count tests were used to compared and diagnose the suspected cases of 254 patients with typhoid fever were presented in Table (1).

The results in the table (1) were showed the diagnosis of typhoid infection for 232 from 254 suspected cases. The PCR and blood culture were showed positive result in 192 case (75.6%) and 124 case (48.8%) respectively, while 117 suspected cases were observed positive (46.1%) according to a titer antibody against somatic (O) and/or flagella (H) antigens of ≥1:160 with WBCs counting test from the 254 suspected cases and 8 cases (40.0%) of healthy persons were observed positive of Widal test whereas 12 cases (60.0%) were observed negative for all tests. Negative test results in blood culture and PCR on blood were obtained for 22 patients with a diagnosis of typhoid fever. The positivity of PCR was found highly significant ($P \leq 0.01$) obtained from blood culture and Widal/WBCs count tests.

Table (1) Three diagnosis methods (PCR, blood culture and Widal / WBCs count tests) on blood of suspected typhoid fever patients

No. of cases with typhoid		PCR	BC	Widal test and WBCs count	Mean duration of illness (days)
Male	Female				
25	17	+	+	+	9.5
28	25	+	+	-	3.7
20	9	-	+	+	8.5
26	36	+	-	-	9.1
20	15	+	-	+	9
6	5	-	-	+	5.3
0	22	-	-	-	7
124	130	(+) = 192/232	(+) = 124/232	(+) = 117/232	7
Control		-	-	+	7
		-	-	-	6.5
		(+)= 0/20	(+)= 0/20	(+)=8/20	7
Probability			1 *vs.* 2 = S	2 *vs.* 3 = S	1 *vs.* 3 = NS

When: (+) is positive result, (-) is negative result, (S) is significant and (NS) is non-significant.

To evaluate the diagnosis of suspected typhoid patients that were showed positive result by the three tests employed (n = 232), the PCR revolved sensitivity of 82.8%, while blood culture and Widal test were 53.45% and 50.43% respectively, in diagnosis of suspected typhoid patients compared to control (Table 2) according to the statistical analysis between these diagnostic methods.

Table (2) The statistical analysis to evaluate of three diagnosis methods (PCR, blood culture and Widal / WBCs count tests) results onto blood of suspected typhoid fever patients (n=254)

Diagnostic methods	Test					
	Sensitivity (%)	Specificity (%)	(PV +)	(PV -)	(LR +)	(LR -)
PCR	٨٢.8	١00.0	١00.0	٣٥.٤٨٣٩	∞	0.١٧
BC	٥٣.٤5	١00.0	١00.0	١٦.٩٢٣١	∞	0.٤٧
Widal/ WBC count	٥٠.٤٣	١00.0	١00.0	16.0584	∞	0.٥

When: (PV+) is positive predictive value, (PV-) is negative predictive value, (LR+) is likelihood ratio for a positive test result, (LR-) is likelihood ratio for a negative test result,(∞) is infinity value.

Discussion:

Typhoid fever is one of the most common infectious diseases in most developing countries. Early and definitive diagnosis of the disease is not only important in relieving patients' suffering, but also critical in avoiding fatal complications such as intestinal perforate. It also makes possible specific treatment at an early stage, which leads to the rapid elimination of the pathogen. Otherwise, and the chronic patient's excreta, especially stool, become a constant source of spread of the disease [(12)].

The positivity of PCR result is agreement with the previous findings in few reports on the application of PCR in the diagnosis of typhoid fever in endemic areas which showed that the similar or lower sensitivity than that observed in the present study.
The reasons of highest sensitivity was found in the present study might be to DNA extraction and a slight modification of the recommended DNA extraction protocol and ensured the presence of at least 1 bacterium in the sample [(13)]. Further, the inhibitors of PCR like hemoglobin were eliminated by repeat lysis of RBCs step when extraction of DNA. Additional to the high specificity and sensitivity of

primer used in the present study to detect of *fliC* gene of flagella of *S. typhi*, when the single round of PCR with ST 1 and ST 2, amplification products of the expected size 458bp were shown only from the extracts of *S. typhi* strains but not from the DNA extracts of other organisms [14]. The PCR resulted in amplified fragments that were visible after agarose gel electrophoresis (Figure 1).

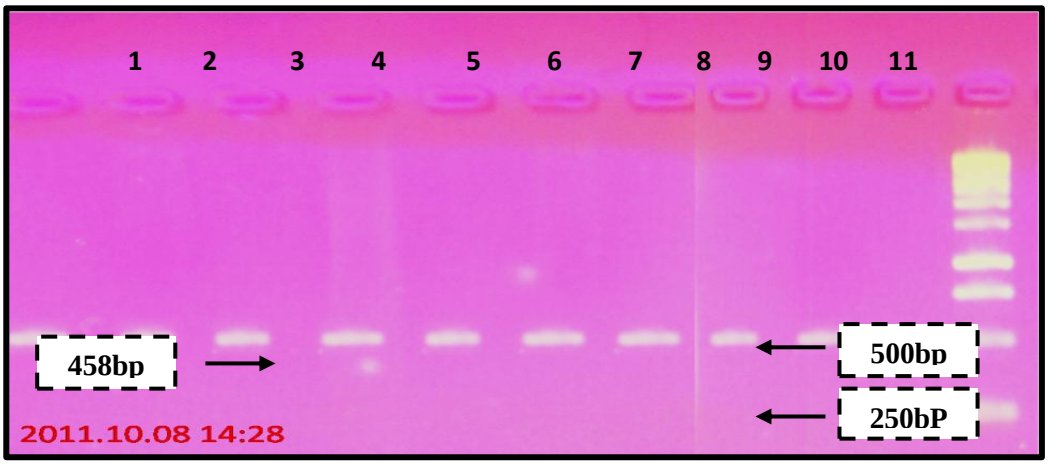

Figure (1) Detection of the flagellin gene of *S. typhi* by PCR. Amplification products of 458bp from the single round of PCR were analyzed by electrophoresis through a 1.5% agarose gel for 90 min. at constant 80 V., Lanes 1 is Positive control DNA isolated from *S. typhi*; lanes 2 to 9, were seen the amplify products with the first round of PCR to DNA extracts sample from suspected typhoid blood patients; lanes 10 is negative control containing master mix without DNA samples; and Lanes 11 is molecular weight marker (1-kb ladder) (DNA ladder).

The negative predictive value result of PCR was revealed clearly compared to the blood culture. As well as, some of the cases that showed positive result exclusively by the Widal test may not be true cases of typhoid, the study finding 70% of these cases that recorded as (PV-) may be less than the actual rate of positivity.

For assigning a test to be of clinical utility, it was recommended that the LR^+ and LR^- of the test should be ≥ 10 and ≤ 0.1, respectively [15]. In the present study, the LR^+ and $LR^-$of PCR were found to be ∞ and 0.17, respectively.

Serologic-Hematology analysis with Widal/WBCs count tests considered a quickly result and made it a deficiency method, that limited value of the sensitivity were titer ≥1:160 compared with that obtained from blood culture and the PCR. Furthermore that value is lower than the sensitivity of 47.5% reported for the Widal test in other studies [14].

The low sensitive of Serologic-Hematology analysis was partial observations by the relative high proportion of tested patients at early stage of the disease who presumably have not developed significant levels of specific agglutinin antibodies because its require 6-12 days to appears after the onset of the disease and can be detectable levels [16], and that reasons could be appearance of 53 case were negative for widal test but it were positive of PCR and blood culture (mean day of presentation of 3.7 days) (Table 1). Moreover, the specificity of the Widal test was high and may be decreases because high levels of antibodies participate in results of reaction [17]. It was important to note that a total of 11 cases and 8 cases were positive by Widal test at this titer but were negatively by PCR and blood culture of suspected typhoid patient and control respectively, these observations due to anamnestic response and could be false positives of Widal test [18].

Blood culture was the standard method for diagnosis of typhoid fever. The isolation rate of *S. typhi* with standard culture techniques is between 40-70% [19]. In this study, blood culture was positive in 124 (48.8%) from 254 clinically suspected typhoid patients and positively blood culture was higher significantly ($P < 0.01$) than that found in Widal/WBCs count tests.

The sensitivity of blood culture was (٥٣.٤5%) and it's less than of PCR, due to various factors such as few numbering of bacteria needed to cause severe infection, which can be as low as 10 cell/ml [12] Hence; positive culture yields were very low and elude definitive diagnosis and this demonstration why the presence of 62 case was negative for blood culture but they were positive of PCR only and 35

case was negative for blood culture but positively of PCR and Widal /WBCs count tests respectively (Table 1). Other limiting factors, are the bacteriostatic effect of antibiotics, nature of culture medium, time of blood collection because the sensitivity of blood culture was highest at first week of the illness and reduced with advancing illness so it could why the presence of 62 cases were negative by Widal test and blood culture but were positive exclusively by PCR, (mean day of presentation of 9 days) (Table 1), in additional to, the host's immune response system, and the intracellular characteristics of *S. typhi* [18]. However, blood culture was time consuming and takes at least 2 to 5 days until the identification of the organism and several factors may contribute to failure to isolate of the organism from blood, including inadequate laboratory media, the volume of blood required for culture, and the presence of antibiotics [20]. From the totaling samples, twenty two cases were negative for three diagnostic methods, that result may be referring to either of false positive of clinical features and/or infection with other species of *Salmonella* such as *S. paratyphi* A [21].

Conclusion:

The observed results suggest that the PCR technique can be used in the early diagnosis of typhoid fever as a considering super standard diagnostic method, which will not only reduced morbidity, mortality, and acquisition of the carrier state but will also limited the transmission of the disease. Furthermore, the blood culture is sufficient to diagnose suspected typhoid patient in the first week and the Widal test was seems to be relevant in the second week of illness at the proposed titer.

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