The comparison of cultures, widal agglutination test and polymerase chain reaction as a diagnostic tool in typhoid fever

Research Article

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Abstract: Typhoid fever caused by *Salmonella typhi*, paratyphi A and B, is an important cause of morbidity and mortality in many developing countries. A rapid and sensitive method for the detection of *S. typhi* is essential for early diagnosis of typhoid fever and effective therapy. In this study 45 febrile patients who were suspected to have enteric fever were enrolled, and the results of blood cultures, widal agglutination tests and Polymerase Chain Reaction in these cases were evaluated. Group I consisted of 11 patients with diseases other than salmonella infections, group II represented 6 patients with positive cultures, and group III represented 28 patients with negative blood cultures negative but who were clinically suspected cases that had a medical history of using variable antimicrobial agents. Two positive PCR results were present; one of them was in culture positive group (16,6%) and the other was in culture negative group (3,5%). In our study widal agglutination tests and cultures were found not to be helpful in differential diagnosis. Although PCR based detection of *S. typhi* is reported to be a sensitive and specific test for the diagnosis of enteric fever, in our study the benefit of this method in the diagnosis of especially patients who were treated with antimicrobial therapy was not clearly determined. Other methods to increase sensitiviy and specificity to levels such as those of real time PCR should be developed and large-scaled studies should be done in endemic and non-epidemic regions.

Keywords: Widal agglutination test • Polymerase chain reaction • Typhoid fever

1. Introduction

Typhoid fever caused by *Salmonella typhi* is an important cause of morbidity and mortality in many developing countries. Typhoid fever was estimated to have caused 21.6 million illness and 216500 deaths globally in 2000 [1]. A rapid and sensitive method for the detection of *S. typhi* is essential for early diagnosis of enteric fever and thus effective and rapid therapy.

Several different techniques are used for the diagnosis of typhoid fever. The gold standard for typhoid fever is isolation of *S. typhi* in the samples of patients including blood, bone marrow aspirates, stool, urine and rose spots. However, blood cultures fail to detect 10-70% of patients with typhoid fever with the different ratios changing due to the amount of blood sampled, phase of the disease, type of culture medium, length of incubation period and prompt antimicrobial usage [2,3]. A second disadvantage of the blood cultures is its requiring at least about 5 days for the isolation [4].

The most commonly used conventional method is the Widal agglutination test. Although its advantages include low cost and easy conductance, this test had limited diagnostic value due to moderate sensitivity and specificity and association with high false-positive and false-negative results [5]. Especially in endemic regions, widal tests were not easy to interpret since cross-

reactions could be seen in other infectious diseases such as malaria and dengue [5]. Also, variable sensitivity and specificity results of the widal tests could be seen in different geographic regions, and different cut-off titers make the test unreliable [6]. The widal test would be also positive quite late in illness Other new tests such as the Tubex test, Typhidot tests and IgM dipstick tests are new techniques but more investigations to evaluate sensitivity and specificity are required [7].

Recently new molecular techniques have been developed and used in diagnosis of infectious diseases including salmonella infections. Polymerase chain reaction (PCR) is a rapid and sensitive method that can amplify one copy of the target DNA of *S. typhi*.

In this study, we have evaluated the results of blood cultures, widal agglutination tests and the PCR technique from children with suspected typhoid fever.

2. Material and Methods

2.1. Patients

Blood samples were taken from the patients with suspected enteric fever who were admitted to Hacettepe University Ihsan Doğramacı Children's Hospital between 1 April 2006 and 1 April 2007 after informed consent was obtained. All the patients had fever for at least 72 hours and at least one of the following complaints, including diarrhea ± vomiting, constipation, abdominal pain, central nervous system complaints and clinical findings including fever, headache, confusion, hepatomegaly, splenomegaly and abdominal discomfort.

Patients were investigated for other etiologic infectious and inflammatory diseases. Patients were regrouped into three groups (Table 1) according to the etiology of fever. Blood samples for cultures, widal tests, PCR urine and stool cultures were obtained from all patients and bone marrow cultures were also obtained from the indicated cases. Laboratory tests including serum hemoglobin levels, white blood cell count, platelet count, peripheral smear, erythrocyte sedimentation rate, serum C-reactive protein, aspartate amino transferase, alanine amino transferase, lactate dehydrogenase levels, protrombin time, activated partial thromboplastin time were applied to patients. For diagnostic purposes bone marrow aspirations, fibrinogen levels, ferritin levels, serum triglyceride levels, total cholesterol levels, abdominal ultrasonography, echocardiograpy were done in indicated cases. The following serological tests were applied, including Ebstein-barr virus, Clamidia pneumonia and mycoplasma pneumonia, cytomegalovirus, herpes simplex virus and brucella, as well as parvovirus B19 DNA if indicated.

2.2. Sampling

Approximately 10 ml of blood specimens was collected from the patients, and 5 ml of the specimen was put into a bottle containing 15 ml bile broth for blood culture. 1 ml was separated for extracting DNA into a tube and rest was separated for Widal agglutination tests.

2.3. PCR

The total DNA from serum samples was purified using the standard phenol/chloroform extraction and ethanol precipitation method as described elsewhere and recommended previously (Sambrook, Cocolin). Twenty pg/ml of each of the primers Salm-3 (5'-GCTGCGCGCGAACGGCGAAG-3') and Salm-4 (5'-TCCCGGCAGAGTTCCCATT-3') were used in a 50 μl PCR mix containing 10 mM Tris-HCl (ph:8.3), 50 mM KCl, 1.5 mM MgCl$_2$ in PCR Reaction Buffer (Roche Diagnostics, Germany), 0.25 mM dNTPs (PCR Nucleotide Mix, Roche Diagnostics, Germany) and 2,5 U Taq DNA polymerase (Roche Diagnostics, Germany) (Cocolin). Thermocycling was performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA) using the program that consisted of 2 minutes of denaturation at 95°C, 35 cycles of 95°C for 90 seconds, 58°C for 80 seconds and 72°C for 2 minutes, followed by a final extension at 72°C for 7 minutes as described before (Cocolin). Expected amplicons of 389 basepairs were visualized in 2% agarose gel after electrophoresis and ethidium bromide staining under ultraviolet light. A strain of Salmonella group D, isolated and identified by biochemical tests were used as a positive control after cultivation on SS and McConkey agar and purification by High Pure PCR Template Kit ® (Roche Diagnostics, Germany). Preparation of PCR mixes and electrophoresis were performed in separate laboratories to prevent cross-contamination.

2.4. Widal agglutination tests

Commercially prepared coloured antigen was used as *S. typhi* O and H antigens. The patients' sera were tested for agglutinins against each of the different Salmonella suspensions and the widal agglutination test against *Salmonella typhi* O antigen titer of 1:160 in the single sera or more was considered positive.

Table 1. Signs and symptoms of cases in groups I, II and III.

Sign and symptoms	Group I	Group II	Group III	Total	Percentage
Fever	11	6	28	45	100
Diarrhea ± vomiting	6	4	16	28	62,2
Constipation	3	2	9	14	31,1
Abdominal pain (abdominal discomfort)	5	5	14	24	53,3
Central nervous system findings (including altered consciousness,meningeal signs, encephalopathy)	1	1	3	5	11,1
Arthritis	1	1	2	4	8,8
Rash	1	2	3	6	13,3
Bradycardia	-	-	1	1	2,2
Rose spots	-	-	-	-	
Splenomegaly	1	-	-	1	2,2
Hepatomegaly	1	-	-	1	2,2
Hepatosplenomegaly	3	2	2	7	15,5

3. Results

3.1. Characteristics of patients

There were 45 patients with prolonged fever and the associated clinical features listed above, including 26 boys and 19 girls with ages ranging from 1 month of age to 17 years (mean 6.45 ± 4.7 years, median age 5.00). Seventy one percent of the patients had diarrhea ± vomiting, 64,4% had abdominal discomfort, 31,1% had constipation, 4,4% had central nervous system findings such as confusion (Table 1).

Overall, the average serum hemoglobin level of the patients was 11,5 ± 1,6 g/dl (median 11,7 g/dl), the average white blood cell count was 10440 ± 3789/mm^3(median 10700 /mm^3), the average platelet count was 368866 ±153000/ mm^3, the aspartate amino transferase level was 50,4 ± 95,6 U/L (median 30 U/L) and the alanine amino transferase level was 34,05 ± 68,89 U/L (median 20 U/L).

Group I consisted of 11 patients whose fever was found to be due to diseases other than salmonella infections, including Kawasaki disease, hemophagocytic lymphohistiocytosis, sepsis due to *E. coli*, osteomyelitis, systemic juvenile arthritis, cholangitis and meningitis. Group II represented 6 patients with positive cultures in which group D salmonella serotype was isolated. Group III represented 28 blood culture negative but clinically suspected cases, in which all patients in this group had a medical history of using variable antimicrobial agents for a two week period.

In group II salmonella group D was isolated in 6 patients, while in groups I and III there was no isolation of salmonella species in the cultures (Table 2).

3.2. Interpretation of Widal-agglutination tests

In group I 4 patients (36,3%) had positive Widal agglutination tests. In the culture positive cases 4 patients (66,7%) had positive Widal agglutination test, whereas in the culture negative group 11 patients (39,25%) had positive widal agglutination test results (Table 2).

3.3. Results of PCR assay

Two positive PCR results were obtained; one of them was in the culture positive group (16,6%) and the other was in the culture negative group (3,5%). The positive result in group II belonged to a patient for whom bone marrow and blood cultures were both positive for *S. typhi* (Table 2).

4. Discussion

Infections due to salmonella infections are an important global health problem. A rapid and sensitive method for detection of *S. typhi* is important not only for effective therapy and diagnosis, but also for prevention of complications and dissemination of outbreaks. Since the clinical features of enteric fever were non-specific and several diseases were considered in differential diagnosis, a new rapid and sensitive method for differential diagnosis of enteric fever was required.

Table 2. Comparison of PCR and blood culture positive for detection of S. typhi in groups I, II and III.

Group	Number of cases	Positive widal agglutination test (%)	Blood cultures positive for *S. typhi* (%)	Amplicons of desirable size present in PCR for *S. typhi* (%)
I	11	4 (36,3%)	-	-
II	6	4 (66,7%)	1	1
-*S. typhi*	1	1	1 (100%)	1 (100%)
-non-typhoidal	5	3	-	-
III	28	11 (39,25%)	-	1 (3,5%)

Diagnosis of typhoid fever is based on primarily on isolation of bacteria in clinical samples especially from blood cultures, bone marrow and sterile body fluids. In our study the ratio of culture positive to negative cases were low, and the probable factors were prior administration of antibiotics and collection of samples 7–10 days after the onset which was associated with second bacteremic phase characterized by low numbers of bacteria in circulation [8,9]. The patients in the culture positive group (group III) had a history of prior antibiotic administration strongly suggesting the negative effect of prior antibiotic usage. Blood cultures were reported to be negative in 30-55% of cases and dependent factors include the amount of bacteria in circulation, the sampling procedures and volume of sampled blood cultures [1].

The second most commonly used and preferred diagnostic tool for typhoid fever is the widal agglutination test, which had limitations such as low sensitivity and limited benefits when one serum sample was obtained [6]. Although in the culture positive group (group II), 66,7% of the patients had positive widal agglutination test results, no significant difference was found between these three groups.

In our study, two positive PCR results were present, one in group II and one in group III; no positive PCR result from the samples taken in group I was present. The oligonucleotide primers used in the study had been described for detection of salmonella species, especially for *S. typhi* infections targeting the invA gene [10]. In our study when further serotyping of the isolated group D salmonella species, one *S. typhi* serotype was recognized in which the PCR test was also positive. The invA gene was reported to be essential for invasion to the epithelial cells, which means when expressed, a higher chance for bacteremia was observed [10] but the specific 389 bp PCR amplicon was reported to be not specific for *S. typhi*. The positive case for desired PCR amplicon was in group II and limited to the case with positive blood cultures for *S. typhi* serotype; this PCR method could be more specific to *S. typhi* than reported before. In our study specificity of PCR methods were found to be higher than sensitivity since it was all

negative in group I. Our study had a limitation because we did not use the DNA hybridization method that could increase the sensitivity of PCR. Although a study using the same PCR method reported before that they were not able to find positive results in clinical samples (in that report the 389 bp PCR amplicon was shown in samples from artificially inoculated rats) [10], we were able to detect bacteremic patients with this method in groups II and III. The low ratio of PCR positive cases in the culture negative group could be due to the presence of inhibitor material such as previously administered antibiotics. Additional methods to increase the sensitiviy of PCR were required. Antibiotics were widely used among typhoid fever patients before diagnosis especially in non-endemic areas, since it mimics a wide spectrum of diseases such as sepsis due to other gram negative bacteria, Kawasaki disease, etc.

In conclusion, although PCR based detection of *S. typhi* is reported to be a sensitive and specific test for diagnosis of typhoid fever, studies concerning the benefit of PCR in the diagnosis of patients who were treated with antimicrobial therapy are not present. In our opinion, PCR based diagnosis of *S. typhi* is a sensitive and specific test as reported before [4], but other methods to increase sensitiviy and specificity such as real time PCR should be performed and large-scaled studies should be done in endemic and non-epidemic regions since multicenteral studies are lacking in the literature.

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