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Short Communication

Utility of PCR in Diagnosis of Problematic Cases of Typhoid

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SUMMARY: Typhoid is a global problem. Conventional diagnostic methods have limitations. The Widal test gives a high proportion of false positive results, and indiscriminate use of antibiotics has reduced the utility of blood culture. Consequently, these procedures are inadequate for diagnosing suspected cases of typhoid that do not present clear-cut symptoms. We previously showed that PCR-based diagnosis of typhoid targeting the flagellin gene has unparalleled specificity. We assessed the utility of this method for diagnosis of problematic cases of typhoid. A comparative study of PCR, blood culture, and Widal test was carried out on 55 cases of suspected typhoid with fever for 3-30 days and possessing an ambiguous clinical picture. A control group comprised of 20 healthy persons was also included. The respective positive results by PCR, blood culture, and Widal test for these groups were 58.2 and 0%, 14.5 and 0%, and 52.7 and 45%. Sensitivity of PCR as compared with that of blood culture was significantly better. We concluded that PCR is much superior to conventional methods and, due to its high sensitivity and specificity, can be of great use for rapid and definitive diagnosis of problematic cases of typhoid.

There are more than 21 million cases of typhoid each year throughout the world (1). In developing countries, due to poor hygienic conditions and inadequate medical facilities, subclinical infection is very common. Many microbiological and serological techniques have been used for diagnosis of these cases. These techniques include blood culture, bone marrow culture, rectal swab culture, urine culture, rose spot culture (2-3); duodenal string culture (4); Widal test (5-6); and ELISA (5).

Widal test and blood culture remain the most common diagnostic procedures because other methods are either invasive or they have failed to prove routinely utile. Widal test is sensitive but highly nonspecific (6,7). Therefore, its effectiveness in diagnosis of varied cases of typhoid is not significant. Blood culture, though highly specific, is helpful only in diagnosis of early typhoid. In such cases, it can only detect 40-45% of positive subjects (8).

Hybridization using DNA probes, the first molecular biology technique used, has 99% specificity but cannot detect less than 500 bacteria/ml (9). However, the advent of PCR technology, already in use for various other diseases, has provided a powerful diagnostic tool. Even 1-5 bacteria/ml are detectable, and results can be available in 1-2 days (10-12).

In developing countries, usually 3-4 days after infection elapse before the patient grows concerned about his or her protracted fever. Such cases can present a challenge to the clinician. The clinical symptoms are usually vague, and routine diagnostic methods have limited utility. This study was designed to evaluate the value of PCR for diagnosis in such cases.

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In this study, suspected cases of typhoid with fever for 3-30 days but an ambiguous clinical picture (only one or two classical symptoms such as enlarged spleen, headache, rose spots, malaise, abdominal discomfort, lethargy, constipation followed by diarrhea, fatigue, delirium, and agitation) were included. Patients represented both sexes and a wide range of ages. The control group consisted of 20 healthy individuals with no history of fever for the previous year. PCR (targeting the flagellin gene), blood culture, and Widal test were performed on samples from these cases to evaluate the relative utility, specificity, and sensitivity of these methods.

Two well-characterized strains of *Salmonella typhi* (STK-1 and STK-2) were used for standardization of PCR conditions, and as reference strains. Extraction of DNA from bacterial cultures and blood samples was performed by the phenol/chloroform method as previously reported (12). For blood culture (performed on two successive days), 2 ml of blood was inoculated into a culture bottle containing 16 ml of trypticase soy broth (Difco, Detroit, Mich., USA) with 0.02% SPS (sodium polyanethanol sulfonate) and incubated at 37°C for 72 h. Using the broth culture, subculturing and biochemical identification was performed by conventional methods (13). Confirmation of isolates was carried out by PCR. Widal test was performed by tube method according to manufacturer's instructions, using reagents provided by Biosystems (Barcelona, Spain).

For PCR, primers reported by Song et al. (11) and modified by Frankel (14), which target the flagellin gene of *S. typhi*, were synthesized on Pharmacia LKB Biotechnology (Uppsala, Sweden), Gene Assembler Special. Oligonucleotides ST1 (5′-TATGCCGCTACATATGATGAG-3′) and ST2 (5′-TTAACG CAGTAAAGAGAG-3′) were used for regular PCR to amplify a 495 bp fragment corresponding to nucleotides 1036-1056 and 1513-1530, respectively, in the flagellin gene of *S. typhi*. For nested PCR, oligonucleotides ST3 (5′-ACTGCTAAAA CCACTACT-3′) and ST4 (5′-TGGAGACTTCGGTCGCGT AG-3′) were used to amplify a 363 bp fragment corresponding

to nucleotides 1072-1089 and 1416-1435, respectively, of the same gene. For regular PCR, 100 μl DNA amplification mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 pmol of each primer, 70 nmol of each dNTP, 1 μl of *Ampli Taq*, 20 μl of template, and distilled water to make up the volume. Using a thermal cycler (Perkin-Elmer Gene Amp PCR system 2400: Perkin-Elmer Corp., Norwalk, Conn., USA), the reaction mixture was subjected to 25 cycles of 1 min each at 94°C, 55°C, and 72°C, followed by heating at 72°C for 7 min. For nested PCR, conditions were the same except that a 1:5 dilution of amplified product was used as the template. PCR was performed in duplicate for each sample. A positive control representing 5 bacteria/ml of *S. typhi* and a negative control without any DNA were also included in each lot. Ten microliters of reaction mixture was fractionated electrophoretically in 2.5% agarose gel containing 0.5 μg of ethidium bromide per ml and was photographed using Eagle Eye (Stratagene, La Jolla, Calif., USA).

It has been reported that a single bacterium of *S. typhi* contains 4 fg of DNA (11). Using this reference, dilutions were made in distilled water from DNA extracted from culture of standard strains STK-1 and STK-2, then used as the template for PCR. The results showed that the regular PCR had a sensitivity of 10^5/ml, whereas nested PCR could detect even 5 bacteria/ml (data not shown).

The patients suspected of typhoid included in this study had experienced fever for 3-30 days, with suggestion of typhoid fever but no clear-cut diagnosis. As shown in Table 1, Widal test was positive in 52.7% (29/55), and blood culture was positive in only 14.5% (8/55) of cases. PCR was positive in 58.2% of cases (32/55), and among these, 14.5% (8/55) were positive for PCR only. Therefore, had the PCR not been performed, these eight cases would not have been diagnosed.

The results of PCR, blood culture, and Widal test performed on healthy persons reporting no recent history of fever are shown in Table 2. This group was used as a negative control.

Table 1. Comparative study of PCR, blood culture, and Widal test in suspected cases of typohoid

No. of cases	PCR	Blood culture	Widal test[1]
6	+	+	+
2	+	+	–
16	+	–	+
8	+	–	–
7	–	–	+
16	–	–	–
55	32(58.2)[2]	8(14.5)	29(52.7)

[1]Widal test was considered positive if titer of antibodies against *Salmonella typhi O* antigen was more than 1:160.
[2]Figures in parentheses indicate percentage.

Table 2. Comparative study of PCR, blood culture, and Widal test in healthy individuals

No. of cases	PCR	Blood culture	Widal test[1]
9	–	–	+
11	–	–	–
20	0	0	9(45)[2]

[1]Widal test was considered positive if titer of antibodies against *Salmonella typhi O* antigen was more than 1:160.
[2]Figures in parentheses indicate percentage.

PCR and blood culture were negative in all cases. However, Widal test was positive 45% of cases.

Typhoid is a major disease in developing countries. Routine efforts are made to treat patients by self-medication facilitated by the free availability of drugs without medical prescription. By the time the clinician is consulted, usually at least 3-4 days have passed. This delay not only makes clinical diagnosis difficult in many cases, but renders conventional diagnostic methods inadequate or useless.

Widal test and blood culture are the most favored techniques for typhoid diagnosis because they can be easily carried out and patient's comfort is not compromised. Widal test is non-specific, and false positive results are very common (15). Blood culture has the promise of diagnosis in the first week of infection, but sensitivity is poor. Less than 10 bacteria/ml can cause severe infection, and are usually undetectable by blood culture (16). At later stages, the sensitivity of blood culture declines even further as a result of some limiting factors such as the host's immune response system and the intracellular characteristics of *S. typhi* (17).

PCR has been reported to detect even 1-5 bacteria/ml with high specificity (10,12). We thought that it would be especially useful for diagnosis of problematic cases of typhoid in which the patient comes late to the clinician and does not have specific clinical symptoms. We selected 55 such cases in which patients had experienced fever for 3-30 days. Widal test, blood culture, and PCR were performed on blood samples from these patients. A control group of 20 healthy individuals having no recent history of fever was also included.

For PCR, we preferred to target the flagellin gene because its hypervariable region VI is unique for *S. typhi* (11,14). There is some controversy over targeting this gene, and the specificity of this method has been discussed in various forums. However, as pointed out by Frankel (14), if a different forward primer is used for first PCR which is derived from the DNA sequence just upstream of primer ST1, together with ST2 as described by Song et al. (11), only *S. typhi* and *S. muenchen* sequences are amplified. The use of ST1, which is *S. typhi* specific, together with ST4 for nested reaction gives 100% specificity. The alternative method, in which the *Vi* gene is targeted, can give false positive results because of the presence of *S. paratyphi C* (10). We were able to improve the sensitivity of our nested PCR to a detection level of less than 5 bacteria/ml (data not shown). Our previous experience with this technique (12) and our experience in this study suggest that it is a very satisfactory method for diagnosis of typhoid.

Our results show that Widal test had no discriminating power, given that 45 and 52.7% of cases of healthy individuals and febrile patients, respectively, showed positive. Respective values for blood culture were 0 and 14.5%, indicating a very high level of specificity but poor sensitivity. PCR values for these groups were 0 and 58.2%. Comparatively, it is quite clear that PCR is much superior to these tests in sensitivity and specificity. The importance of PCR in diagnosis of problematic cases of typhoid is further highlighted by the observation that eight (14.5%) cases were positive only for PCR, and these cases would been missed by either of the other two methods.

Generally, there have been some reservations about the use of PCR for diagnosis of typhoid. However, our present study clearly shows that targeting the flagellin gene by careful selection of primers makes it an excellent tool for diagnosis of problematic cases of typhoid. Given that PCR-based diagnosis of infectious diseases like tuberculosis and hepatitis B and C

has proven its value in recent years, the diagnostic power of PCR for difficult cases of typhoid, a very serious health problem in developing countries, holds great potential for use.

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