Validation the Best, Most Accurate and Reliable Assay for Detection of Typhoid

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Abstract

Typhoid fever is becoming an ever increasing threat in the developing countries. Cultivation of bacteria and serology (especially Widal test) give unacceptable levels of false-negative and false-positive results, respectively.

The aims of present work is development of a PCR assay that can target specific multiple genes for rapid detection of Salmonella enterica serovar Typhi (*S. typhi*) .(which has 100% specificity for *Salmonella typhi*) was compared with Widal test and *Salmonella typhi* IgG\IgM rapid test as well as *Salmonella typhi* IgG and IgM ELISA test during the first week of illness of 50 suspected cases of typhoid.

PCR primers for invasion, O, H and Vi antigen genes, invA, prt, and viaB were designed and used for the rapid detection of *S. typhi* by multiplex PCR.

The respective figures of positivity for PCR, Blood culture, Widal test and *S. typhi* IgG\IgM rapid test and *Salmonella typhi* IgG /IgM ELISA were 66%, 52%, 46%, 42% and 48% respectively. A control group of 20 healthy persons gave figures of 0%, 0%, 40%, 0% and 0% respectively.

The present study conclude that this PCR-based technique is not only absolutely specific, but also very sensitive and, therefore, much superior to Widal test, Blood culture *S. typhi* IgG\IgM rapid test and Salmonella typhi IgG/IgM ELISA tests for the early diagnosis of typhoid.

Introduction

Typhoid (enteric) fever is still a common disease in many developing countries as well as it is a major health problem throughout the world. Globally, there are more than 21 million cases of typhoid each year, with more than 700,000 deaths. It is especially prevalent in developing countries. Early detection of the disease is very important for its control, but unfortunately, current definitive diagnostic tests are inadequate. [1,2,3].

Diagnosis is made by any blood culture, bone marrow or stool cultures and with the Widal test (demonstration of salmonella antibodies against antigens O-somatic and H-flagellar)., urine culture, rose-spot culture, duodenal string culture, ELISA and immunofluorescence. Widal test and blood culture remain the only universally practiced diagnostic procedures, because other methods are either invasive, have failed to prove their utility, or are expensive [4].

Widal test is quite sensitive but has become highly nonspecific, poses some disadvantages in endemic areas. Previous exposure to *S typhi* or antigenically related Gram negative bacilli and vaccination against typhoid can result in raised titres in the absence of a current infection. In contrast, a poor antibody response to either the “O” or “H” antigen (or both) can occur in some patients. Hence, the Widal test often leads to confusion and, on occasions, to misdiagnosis of other febrile illnesses as typhoid fever [5,6].

Another shortcoming of the Widal test is that it becomes positive only in the second week of illness, so its value for early detection of the disease is limited [7].

Blood culture is positive in the first week but its utility is restricted by the very

low numbers of bacteria causing severe disease (which may be less than 10/mL). As a consequence, blood culture can detect only 40%-45% of cases, and even if antibiotic treatment has not been administered, the rate of detection is not more than 70%. Molecular techniques, as is the case in blood culture, target the pathogen itself (not antibodies produced against it), so they can be useful for the early detection of the disease [8].

Clinically suspected cases of typhoid who had had fever for less than three days and had not received any anti-typhoid therapy were included in this study.

While the blood culture tests though time-consuming is considered the gold standard in typhoid fever diagnosis [8], so the sensitivity of the MPCR assay with the given primer sets (By targeting three genes in *Salmonella typhi*), Widal test, *Salmonella typhi* IgG/IgM rapid test and *Salmonella typhi* IgG/IgM ELISA tests were performed on samples from these cases and next calculated and compared to the sensitivities of the blood culture test, which are the conventional means of diagnosis for typhoid fever, to evaluate their relative utility, specificity and sensitivity.

Materials and Methods

Patients

Fifty patients included in this study had fever for less than 3days, most of them are Widal positive, had had no anti-typhoid treatment, Members of both sexes representing all ages were included, all serum samples were collected from Mirjan educational Hospital during the summer of 2012. The control group constituted 20 healthy individuals with no recent history of fever.

Blood culture test. Patient blood samples (2.5 ml) were drawn and added to brain heart infusion medium. This medium was then incubated at 37°C for 24 h and later streaked onto MacConkey agar plate. If the colony did not ferment lactose, further oxidase test and slide agglutination test were done. The colonies that gave negative results by the oxidase test and a positive result by the slide agglutination test were further confirmed through biochemical tests where *S.* typhi is indole negative, and urease negative and does not ferment mannitol [9].

Widal test. Rapid slide test for qualitative in vitro determination of antibodies in serum against *Salmonella* Typhi O and H antigens and/or *Salmonella* Paratyphi A(H) and B(H) antigens were done using TyDAL kit (Arsitha Diatech) following the manufacturer's instructions.

***OnSite* Typhoid IgG/IgM Combo Rapid Test-Cassette**

The *OnSite* Typhoid IgG/IgM Combo Rapid Test (Biotech, USA) is a lateral flow immunoassay for the simultaneous detection and differentiation of anti-*Salmonella typhi* (*S. typhi*) IgG and IgM in human serum, plasma or whole blood. It is intended to be used as a screening test and as an aid in the diagnosis of infection with *S. typhi* [10].

***Salmonella typhi* IgG and IgM ELISA**

S. typhi specific IgM and IgG antibodies were estimated according to the manufacturer instructions (Calbiotech, a life Science company), the positive IgG & IgM was calculated depend on finding the index value, more that 0.08 index value represent as positive cases.

DNA Extraction and Identification

One mL of blood containing 20 mM potassium EDTA as anticoagulant was centrifuged at 10,000 rpm for 5 minutes. Plasma was separated for serology. One mL of lysis buffer (0.2% Triton X100 in Tris HCl pH 8.0) was added to the pellet. The mixture was gently aspirated several times to effect hemolysis. The tube was centrifuged at 12,000 rpm for 6 minutes, the supernatant was discarded, and the procedure was repeated once. The pellet was washed once with distilled water. After the removal of the supernatant, the pellet was resuspended in 20-30 µL of distilled water. The tubes were sealed, kept

in boiling water for 20 minutes, and brought back to room temperature before being used as a template for PCR.

DNA concentrations were measured with nano-drop DNA concentration detector at 260 nm (Thermo Scientific, U.S.A.).

Primers for conventional PCR

Three pairs of primers were reported by (Kumar, 2005) , synthesized by GenScript company, USA, and the quality of primers confirmed by BLAST (Basic Local Alignment Search Tool) The specificity of these sequences for three genes was confirmed by BLASTx search on Gen Bank (http://blast.ncbi.nlm.nih.gov).

Table. 1 Primers for multiplex PCR amplification of *S. Typhi*

amplification of *S. typhi* Primer sequence 5 to 3	Target gene	amplicon size(bp)	position of primers	Accession No
InvAF CGAGCAGCCGCTTAGTATTGAG	Inv A	881	978–999	U43273
InvAR CCATCAAATTAGCGGAGGCTTC			1858–1837	
ViaBF CACGCACCATCATTTCACCG	viaB	738	1858–1837	D14156
ViaBR AACAGGCTGTAGCGATTTAGG			902–882	
PrtF CGTTTGGGTTCCTTGGATCACG	prt	369	383–404	M29682
PrtR CTATAATGGCGGCGGCGAGTTC			751–730	

PCR amplification and analysis of PCR products

Multiplex PCR was carried out in 25 µl reaction containing 0.3µ mol ¯1 of prtF and prtR primers, 0.2 µmol¯1 of all other primers, PCR master mix product *(AccuPower* PCR PreMix) from Bioneer, USA was used , with 2.5 µl of template DNA, the final volume was completed to 25 µl by ddwater. PCR was taken through 42 cycles in VERTI (Thermal cycler, Applied Biosystem, USA) at 94◦C for 30 s (denaturation), 60◦C for 90 s (annealing) and 72◦C for 2 min (extension). Gradient temperature from 57 to 64◦C was initially used before finalizing 60◦C as annealing temperature. The DNA was denatured for 4 min at 94◦C in the beginning and finally extended for 5 min at 72◦C. PCR products were analysed in 1.5% agarose.

Results

Multiplex PCR (mPCR) was optimized using the profile as outlined in Materials and methods. The annealing temperature of 60◦C was finally selected though bands were visible at all the temperatures. The concentration of prtF and prtR primers was increased to 0.3 µmol ¯l)1 because a faint band was seen at 0.2 µmol ¯l). Analysis by blast search indicated significant specificity of the primers for the desired gene sequences.

Comparison of PCR, Blood Culture ,Widal Test, IgG/IgM rapid test and IgG/ IgM ELISA tests in Healthy individual.

This group had had no history of fever for the last six months. It was used as a negative control. PCR , blood culture, IgG/IgM rapid test and IgG/ IgM ELISA tests were negative in all 20 cases. Widal test (with a titer of more than 1:160 for *Salmonella typhi* O antigen) was positive in as many as 8 (40%) persons. The results are given in Table -1.

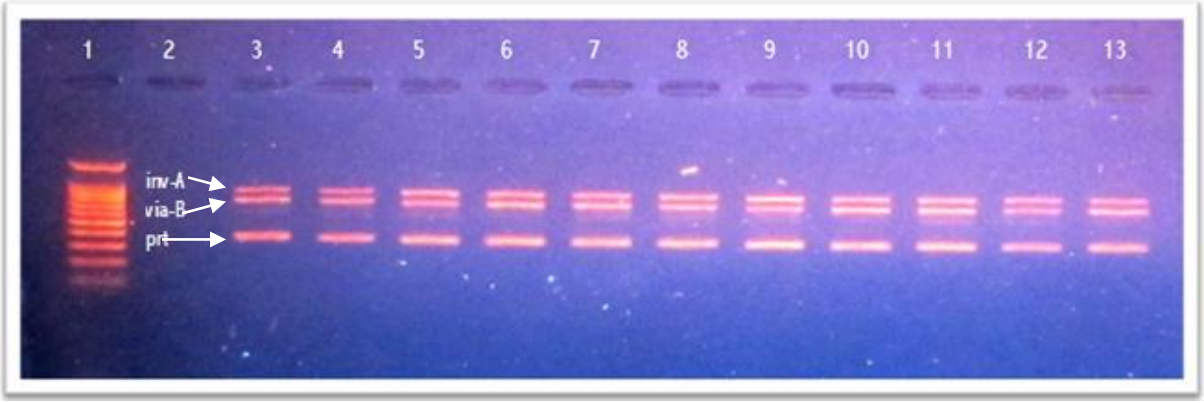

Figure 1: Agrose gel electrophoresis of M PCR products. Lane 1shows the profile of 100 bp marker, lane 2– negative control, Lne 3-posative control, lane 4 to 13 shows *Salmonella typhi* positive cases .

Comparison of PCR, Blood Culture , Widal Test, IgG/IgM rapid test and IgG/ IgM ELISA tests in Suspected Cases of Typhoid.

The patients included in this group represented both sexes and all ages. They had suspected typhoid fever of less than three days' duration and had not taken any anti typhoid drug. The results are given in Table 2. Out of 50 cases, 8 (16%) were positive for all five parameters; 3 (6%) were positive for PCR , blood culture and IgG/ IgM ELISA tests; 10 (20%) showed positivity for PCR and Widal test, *Salmonella typhi* IgM for both rapid and ELISA tests but not blood culture. Both PCR and blood culture were positive in 12 cases (24%), whereas only Widal test was positive in two patients (4 %). Three (6%) patient was negative for PCR but positive for each blood culture ,Widal test, IgG rapid test and IgG/ IgM ELISA. There were 12(24%) cases which were negative for all five parameters.

Table 1. Comparative study of PCR, Blood culture, Widal test , rapid IgG/IgM test, IgG/IgM ELISA assay in 20 healthy individuals.

Cases	PCR	Blood culture	Widal	IgG/IgM rapid test	IgG/ IgM ELISA assay
8	-	-	8	-	-
12	-	-	-	-	-
Total:20	0	0	40%	0	0

Table 2. Comparative study of PCR, blood culture , Widal test rapid IgG/IgM test, IgG/IgM ELISA assay in early diagnosis of typhoid .

Cases(%)	PCR	Blood culture	Widal	IgG/IgM rapid test	IgG/ IgM ELISA
8 (16%)	+	+	+	+/+	+/+
3 (6%)	+	+	-	-/-	+/+
10 (20%)	+	-	+	-/+	-/+
12 (24%)	+	+	-	-/-	-/-
3 (6%)	-	+	+	+/-	+/+
2 (4%)	-	-	+	-	-/-
12 (24%)	-	-	-	-	-/-
Total:50	33 (66%)	26 (52%)	23 (46%)	21 (42%)	24 (48%)

Positive PCR appear as the presence of highly specific of three genes bands on agarose as in Fig-1.

Positive Widal test is a titer of 1:160 or above for *Salmonella typhi* O antibody.

Positive IgG/IgM rapid test: In addition to the presence of C band, if T1 or T2 or both bands are developed, the test indicates for the presence of anti- *S. typhi* IgM or IgG or both IgM+IgG the results are positive.
Positive IgG /IgM ELISA is a 0.08 index value or above.

Table 3. Comparison of PCR, blood culture and Widal test, IgG/IgM rapid test and IgG/IgM ELISA for different groups of study.

Cases	PCR (%)	Blood culture (%)	Widal (%)	IgG/IgM rapid test(%)	IgG/ IgM ELISA(%)
Control group I (healthy persons)	0	0	40	0	0
Typhoid expected patients	66	52	46	42	48

Table-4:Quality management of the assays comparing with a standard test (blood culture test) for the diagnosis of Typhoid.

Assay / Validation	**PCR**	**Widal**	**IgG/IgM rapid test**	**IgG/ IgM ELISA**
Specificity Index Sp. I	100%	50%	58%	50%
Sensitivity Index Se.I	88%	42%	42%	53%
Diagnostic specificity D.Sp.	100%	66%	70%	70%
Diagnostic Sensitivity D.Sc.	89%	63%	63%	68%

Specificity index(SP.I): is the percentage of avidity of antibody to react with its specific antigen; SP.I= $(TN - FP) \div TN$
Sensitivity index(Se.I): the lowest concentration of pathogen can be detected by the assay; Se.I= $(TP - FP) \div TP$
diagnostic sensitivity (DSn): probability of correctly identifying true positive (infected or diseased) animals
DSn = $TP \div (TP + FN)$
diagnostic specificity (DSp): probability of identifying normal or true negative animals DSp $TN \div (TN + FP)$
false positive (FP): identification of a normal subject as test positive
false negative (FN): identification of an infected or diseased subject as test negative
true positive (TP): identification of a diseased or infected subject as test positive
true negative (TN): identification of a normal subject as test negative
[11].

Discussion

Typhoid fever is one of the most common infectious diseases in 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 perforation of the intestines. It also makes possible specific treatment at an early stage, which leads to the rapid elimination of the pathogen. Otherwise, the patient's excreta, especially stool, becomes a constant source of spread of the disease [1].

Blood culture is a more practical albeit less sensitive alternative to bone marrow culture. However, it is not always available and, when it is, it takes 2 to 3 days. As a result, diagnosis may be delayed or overlooked and patients without typhoid fever may receive unnecessary and inappropriate antimicrobial treatment. The low level of bacteria in the sample may set an impenetrable practical barrier which

may only be circumvented by purification or enrichment technology [2].

For these reasons, in developing countries typhoid rapid antibody tests can facilitate diagnosis and disease management, however its sensitivity is lower than blood culture test [12].

The study showed the percent of IgG/IgM ELISA was higher than the percent of detection by rapid tests as well as Widal test for detection typhoid(48%, 42% ,46% respectively). The results of other study confirm that the ELISA-Ty test has a high reliability for the detection of typhoid fever based on the finding of a degree of diagnostic sensitivity as high as 95.45% and 90.91% for respectively IgM and IgG [4].

In this study, a multiplex PCR assay was developed for rapid detection of *S. typhi* from early suspected Typhoid patients, offers highly specific, sensitive and reasonably quick diagnostic modality.

The study shows high percent for detection of typhoid by MPCR (66%). The results are given in Tables 1 and 2. Widal test results were insignificantly different from the healthy group (40% and 46%, respectively).

The assay targeted the amplification of Three genes instead of one as in many other studies. The genes viaB and prt encode for the synthesis of Vi (capsule) and O (LPS) antigens respectively. These antigens form the basis of classification for Salmonella by Kauffman –White scheme. The gene invA, along with other invasion genes, is responsible for invasion of epithelial cells and has been reported to be present in all salmonellas. The gene prt is part of rfb cluster of genes and encodes for CDP paratose synthase which converts CDP-4-keto-3,6- dideoxyglucose to CDP paratose. This gene is present in serovar Typhi. The presence of all three genes were used in diagnosis *S. typhi* [9].

The study showed that the multiplex PCR is direct highly specific and sensitive than the other serological tests(Widal, IgG/IgM rapid test and IgM/IgG ELISA testes) because it target the pathogen itself rather than targeting antibodies, so it can detect typhoid in early stage when IgM and IgG are not developed yet.

Conclusions and Recommendation

The present study conclude that M PCR is much superior than blood culture , Widal test, IgM/IgG rapid detection kit and anti *Salmonella typhi* IgM/IgG ELISA kits, I thought that M PCR might be an excellent tool for the early diagnosis of typhoid. It has great discriminating value due to its very high sensitivity and specificity. Therefore, it can be of singular importance for the detection of early cases of typhoid, which is not only important for the treatment of patients but is also necessary for control of the disease. Due to the need for extensive infrastructure and specialized skills, the PCR facility cannot be made available everywhere, especially in developing countries. Nevertheless, due to its high sensitivity and specificity, as demonstrated in this study, it needs to be made available to everyone by establishing collection centers which can send.

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