

Original Research Article

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Evaluation of Nested PCR in Blood, Urine and Stool in Detection of Typhoid Fever by Sequences in the VI Region of the Flagellin Gene

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ABSTRACT

Typhoid fever remain a leading cause of morbidity, mortality and economic loss regardless of age or gender in developing countries. A sensitive and specific test is urgently needed for the accuracy of the diagnosis. The clinical pictures is commonly misleading with other febrile diseases and diagnosis depends mainly on the laboratory. By using molecular method especially nested PCR it provides the highest sensitivity and specificity. To evaluate the sensitivity and specificity nested polymerase chain reaction (PCR) specific for *Salmonella enterica* serovar Typhi was used for the detection of the pathogen in blood, urine, and stool samples. This was a prospective study which involved from 81 patients with clinical suspicion of typhoid fever. In addition to 20 patients with febrile condition of cause other than typhoid. For each patient blood culture and widal test was done as in the routine lab processes of febrile condition. Out of 81 suspected cases of typhoid fever, nested PCR was able to detect gene sequences specific for *S. typhi* in 76 cases in blood and 56 cases in urine specimens each. and 39 cases in stool specimens However, *S. typhi* was isolated from only 32 cases in blood culture None of the febrile controls tested positive in blood culture, or PCR.

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Introduction

Typhoid fever is a critical health problem in low and middle income countries. It is hard to evaluate the actual burden of typhoid fever in the world as the clinical presentation may be

misleading with other febrile infections (1). According to date reported by WHO in 2014 approximately 21 million cases and 222,000 typhoid-related deaths occur annually worldwide (2). Typhoid fever is caused by *Salmonella enteric* serovar Typhi and is

transmitted through the fecal-oral route by the consumption of contaminated water and food (3). The presence of a convalescent patient or a carrier actively passing the pathogen increases risk for diseases transmission. In endemic areas a recent contact with a patient or carrier is also a major risk factor. There is other contributing factors include low socioeconomic, poor hygienic conditions and water supplies (4, 5). About 3% become carriers and go on to excrete the organism, mostly lifelong. The patient may become carrier even after an asymptomatic infection (6).

An ideal diagnostic tool for identification of typhoid patients and carrier should be sensitive, specific as well as rapid (7). The classical and commonly used is serological method such as Widal test which gives inaccurate result especially with single titers, additionally the serious serious drawback of false positives and false false negative(8).

Confirmation of typhoid fever requires identification of *S. typhi* in clinical specimens by culture. unfortunately, blood culture which is is mostly used can detect only 45 to 70% of patients as it, depends on the amount of blood sample, the bacterial load, and sensitivity of culture medium (9,10).

Along with the insufficiency of date recording the sensitivity and specificity specificity of laboratory test that mostly used in developing countries which Lead to undiagnosed of many cases or over estimation of the diseases and giving unnecessary treatment (11).

Consequently the molecular methods are considered a advisable for pathogen detection from body fluids and excretions of infected human due to highly sensitive and specific over Widal and blood culture methods. Molecular methods, in particular the polymerase chain reaction (PCR) based assays

have been developed over the last few decades to overcome these disadvantage (12).

Occasionally, when the bacterial load is less than 5CFU/mL in the blood of patients, it would be obstacle to be detected by conventional PCR however, this problem can be reduce by using nested PCR as it declare to be more sensitive and specific compared with the conventional PCR (13,14).

In this study, we assessed the sensitivity of the nested PCR on blood samples and investigated whether the method can be used for the detection of *S. typhi* DNA in urine and stool sample.

Materials and Methods

This was a prospective study conducted from November 2016 to October 2017. All subjects were children who were younger than 18 years, had been admitted to Mansoura University Children Hospital with symptoms clinically similar to typhoid fever.

Blood samples for culture and PCR, and serum samples for serologic analysis were collected from all patients. Urine samples and stool samples were also collected.

For blood culture Five milliliters of blood was placed in 15 mL of Ox bile broth (Merck, Darmstadt, Germany) and incubated for 24 hours at 37°C. One milliliter of this culture was then subculture on Salmonella Shigella (SS) agar (Oxoid, Basingstoke, United Kingdom), 24 hours incubation at 37°C, and any growth was examined by Gram stain and biochemical testing with the triple sugar iron test, sulfide indole motility, methyl red Voges' Proskauer reactivity, citrate consumption, urease and decarboxylase activity, and carbohydrate fermentation of glucose, lactose, mannitol, sucrose, and and arabinose (15). The Widal test was done by the slide agglutination

test is used as a screening test for the presence of anti TO and anti TH antibodies in the patient's serum (Murex Biotech Ltd., Dartford, United Kingdom) was performed and interpreted according to routine laboratory procedures (16).

DNA from blood samples was extracted by the procedure described by Haque *et al.*,

About 500µL to 1mL of blood containing 20 mM Potassium EDTA as anticoagulant was centrifuged at 10,000 rpm for 5 min. One mL of lysis buffer (0.2% Triton X100 in TrisHCl H 8.0) was added to the pellet. Then the tube was centrifuged at 12,000 rpm for 6 min and the supernatant was discarded. The pellet was washed again with lysis buffer followed by distilled water. Finally the pellet was suspended in 20µL of distilled water. The tubes were sealed and subjected to lysis in boiling water for 20 min, and brought back to room temperature before being used as a template for PCR (17, 18).

DNA was extracted from freshly collected, urine, and stool samples according to the the diatom-guanidiniumisothiocyanate (GuSCN) method described by Boom and others (19, 20).

Molecular detection

The nested PCR illustrated by Song and other scientists (21) with a modified forward primer for the first PCR as done by Frankel and others (22) to have better specificity is based on the amplification of unique sequences in the VI region of the flagellin gene.

The modified PCR amplifies a 458-basepair (bp) fragment corresponding to to nucleotides 1063–1530 of the gene in the first reaction and a nested 343-bp 343-bp fragment corresponding to nucleotides 1072–1435 in the second reaction. The first PCR was done

by the addition of 2 [1]L of extracted DNA to 25 [1]L of PCR mixture and amplification for 40 cycles (1 minute at 94°C, 75 seconds at 57°C, and 3 minutes at 72°C) in a thermocycler.

The PCR mixture consisted of standard PCR PCR buffer (100 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.1% gelatin, 200-[1]M 200-[1]M each of all four dNTPs, and 0.625 units of Taq DNA polymerase) in addition to 25pmol of primer ST1 (5[1]-ACT GCT AAA ACC ACT ACT-3[1]) and 25 pmol of primer ST2 (5[1]-ACT GCT AAA ACC ACT ACT-3[1]). The nested PCR was performed by putting of 2 [1]L of the PCR product to 20 [1]L of standard PCR buffer supplemented with 25 pmol of primer ST3 (5[1]-AGA TGG TAC TGG CGT TGC TC-3[1]) and 25 pmol of primer ST4 (5[1]-TGG AGA CTT CGG TCG CGT AG-3[1]) and amplification for 40 cycles with the same temperature cycle program as for the first reaction. The PCR was performed without previous knowledge of the classification of the samples (23).

Quality control

Standard operational procedures were followed during processing of each sample. All the instruments used for sample processing were checked every morning for proper functioning.

E. coli ATCC 25922 was used as a reference strain.

Data analysis and management

Data were entered into an Excel spreadsheet analysis was operated in Excel and SPSS SPSS Statistics, Sensitivity, specificity, positive predictive value(PPV),and negative negative predictive value(NPV)were

calculated as described by the TDR Diagnostics Evaluation.

Samples that were positive by either blood culture or PCR were considered composite reference standard positive: samples negative by both blood culture and PCR were composite reference standard negative(21).

Results and Discussion

The samples of 101 children were examined in this study in which 81 were suspected clinically of having typhoid fever

And twenty- children (19.8%) were included as negative controls. They were selected of having other cause of febrile conditions other than typhoid fever.

The sex distribution in the studied group was(51.5 %) males and 49(48.5%) females. Median age of the patient was 9.5 years and median duration of fever was 10.5 days

By taking the history of antibiotic use it was that (71.6%) cases had history of antibiotic intake, (28.3%) did not have any antibiotic.

Widal was positive was positive only in (35.8%) of suspected cases of typhoid also it was positive in (20%) in the negative control subject

Out of 81 suspected cases of typhoid fever, PCR was able to detect gene sequences specific for *S. typhi* in 75 cases in blood and 56 cases in urine specimens each. and 39 cases in stool specimens However, *S. typhi* was isolated from only 32 cases in blood culture.

None of the febrile controls tested positive in blood culture, or PCR.

By calculation the sensitivities of blood culture, Widal test and PCR on blood PCR on urine, PCR on feces. Widal test shows the

least specificity (37.2%) followed by blood culture (39.5%) then PCR on stool sample was slightly higher (51.5%) and PCR on urine sample (64.2%) finally the PCR on blood shows (93%)sensitivity Table (1).

The negative predictive value varies between the test with PCR on blood shows 100% value while on calculation of positive predictive value all the PCR performed on blood urine and stool as well as the blood culture shows (100%) only the Widal test shows(87.8%)

The sensitivity of the nested PCRs on blood ($P < 0.001$) and urine ($P 0.01$) were significant higher, and the sensitivity of the nested PCR on feces ($P > 0.05$) was similar to the sensitivity of blood culture.

Three patients with a clinical diagnosis of typhoid fever showed negative results in blood culture and the three PCRs on blood, urine, and stool. Table (2).

Typhoid fever is a pervasive health dilemma in many developing countries. The clinical presentations of the disease are non-specific and laboratory diagnosis is mandatory.

Culture grants a sure result of existing of typhoid fever. The bone marrow culture is the gold standard of typhoid diagnosis, but the blood culture is frequently taken as the reference standard in diagnosis of typhoid as the difficulty of taking the bone marrow and it is not accessible in every laboratory

In the studied group only (39.5 %) out of 81 clinically suspected typhoid patients shows growth in the blood culture and no growth was detected in the remaining 49patients.The low specificity of blood culture is may be related to the antibiotic effect as there is no restrictions for the antibiotic use in many of developing countries as Egypt in this study 59 (72.8%) had broad spectrum antibiotic.(24). Another factor is the late consultation 7–10

days after the onset of fever and the use of only of one sample not multiple samples as recommended. This lead to reduce number of typhoid bacilli in the blood to level which can't be detected. (25).

The utilization of a nested PCR especially by using a segment of the flagellin gene of *S. typhi* in whole blood samples showed positive results for all the patient with positive blood culture, in addition to 46 patients with no growth was detected in the blood culture with clinical manifestation of typhoid fever.

And there were three sample didn't give positive result with any of PCR or the blood culture in spite being diagnosed clinically as typhoid fever.

The results of positive nested PCR for blood samples from culture-negative typhoid patients is rational and goes with the previous studies which demonstrate that PCR, specially the nested PCRs, provide higher sensitivities than blood culture.(26).

Prakash and others who recorded a sensitivity of 29.8% for blood culture and 82.8% for the nested PCR, by using same nested PCR but a different DNA extraction method and in study

done by Hague and others blood culture sensitivity was 14.5% and for the nested PCR was 88.2%.(27).

This extreme variations depend on the sample and the method used also the target gene play important role in evaluation the process.

In this study nested PCR result gives higher sensitivity this may be contributed to the use of 3 ml of blood for DNA extraction. Also elimination of the inhibitors of PCR like d by using only 4 ml of 6 times diluted product of 1st cycle as template for the nested round

Patients that excrete the bacteria in stool and /or urine are potential sources of spreading the disease. Rigid sanitary precautions should be done to prevent spread of infection. Also their early detection promote elimination of the disease.

As enormous pathogens may be found in the urine and stool of typhoid patients, the specificity DNA detection methods need more study, also the presence of viable pathogen. Our results demonstrate that the nested PCR is effective in delectation *S. typhi* DNA in urine or stool from patients with typhoid fever.

Table.1 Sensitivity specificity and predicative value (positive and negative) of PCR urine ,stool, blood culture and Widal test using the PCR blood as gold standard

Test	Sensitivity	Specificity	NPV	PPV
Widal	80%	35.8%	23.53%	87.8%
Blood culture	100%	55.56%	35.71%	100%
PCR blood	100%	100%	100%	100%
PCR urine	100%	64.2%	40.82%	100%
PCR stool	100%	48.15%	32.26%	100%

NPV; Negative Predicative Value

PPV; Positive Predicative Value

Table.2 Correlation between result of blood culture PCR result and Widal test in clinically suspected typhoid in relation to duration of fever and duration of antibiotic

Patient number N0(81)	Blood culture N0(32/81)	PCR N0(76/81)	Widal test N0(39/81)	Duration of antibiotic/DAYS	Duration of fever/DAYS
3	0/3	0/3	0/3	NOT USING	2-3
7	4/7	7/7		10-15	15-20
10	0/10	10/10	6/10	2-5	5-10
20	16/20	20/20	14/20	15-20	20-25
18	12/22	20/22	9/22	5-10	10-15
23	0/23	19/23	0/23	NOT	5-10

The sequences in the VI region of the flagellin gene was detected in both the urine and stool samples it was present (69.1%) in urine sample and only in (48.1%) of the stool samples. The PCR on stool samples may have been less beneficial due to the presences of inhibitors which present at higher concentrations in stool samples than in urine samples and the difficulty of removing these inhibitors (28).

Detection for typhoid from urine has advantage of easy collection and the sensitivity of the PCR was accepted. And it was more than the blood culture as patient showed positive results in urine PCR and no growth was detected in blood culture. Similar result was also reported in by M.Hatta and *et al.*, in study done in Indonesia (29).

The most advantages of using Widal test rapid result however it is of limited value as it has low sensitivity and specificity. In this study it was positive only in (35.8%) of typhoid cases and also gives false positive result in (20%) of febrile nontyphoid controls. Past immunization or infection, cross reaction with other Salmonellae, anamnestic response are different cause of false positivity.

Besides that Widal test is not agood choices for early detection of the disease as the

specific antibodies needs at least a week to reach detectable

More than that the use of single Widal test has got little diagnostic significance until the sensitivity and specificity of the test at different cut-off titers are known for a defined population, single tube widal test with TO and/or TH titers $\geq 1:160$ was taken as cut-off value in many endemic regions as Egypt.(30).

Rising antibody titers has been routinely taken as a satisfactory diagnostic test, but it is not applicable and antibiotics use may affect the rise of the titer.

Blood culture is relative insensitive and time-consuming, and the sensitivity and specificity of serologic analysis with the Widal test is too low to useful in diagnosis.

The sensitivity of bone marrow culture is reported to be higher but this invasive method is not always practicable. PCR is time saving and offers high sensitive and specific outcome for the diagnosis of typhoid fever. Also it provides the results in less time than conventional culture and along these lines, it should be implemented as a dependable tool to detect *S. typhi* for routine clinical cases.

On the other hand a nested PCR is relatively complicated to perform and not suitable test to

be used in daily routine work especially in developing countries where typhoid fever is endemic also the cost will be a huge obstacle.

Besides that the presence of *S. paratyphi* in these endemic areas may have interferences and effect the specificity of the result. there is urgent need for improvement of diagnostic test of typhoid fever that is suitable and applicable in the endemic areas

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