ORIGINAL ARTICLE

Evaluation of Real Time Polymerase Chain Reaction for *Salmonella* Invasion Gene A and *Salmonella* Tetrathionate Respiration Gene as a Diagnostic Test for Typhoid Fever

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ABSTRACT

Background: *Enteric (typhoid and paratyphoid) fever is a serious systemic disease caused by Salmonella enterica (S. enterica) that needs appropriate microbiological diagnosis mainly with atypical clinical signs due to antibiotics misuse. In Egypt, there are insufficient data about molecular detection of enteric fever.* ***Objectives:*** *To evaluate the quantitative real- time polymerase chain reaction (qPCR) for Salmonella invasion gene A (Inv A) and Salmonella tetrathionate respiration gene (ttr) of S. enterica in typhoid fever patients.* ***Methodology:*** *This was a case-control study that included 100 patients with typhoid fever and 100 control subjects. Ten ml blood were obtained for blood culture, Widal testing and enrichment on tryptic soya broth (TSB) with bile. Positive blood culture was sub cultured, identified and serotyped for isolation of S. enterica. Finally, qPCR was applied for detection of Inv A and ttr genes from blood samples and after enrichment.* ***Results:*** *Blood culture was positive in 51% and Widal test was positive in 70% of patients. Combination of Widal and/or blood culture increased their sensitivity, specificity and accuracy to 82%, 89.2% and 85.5% respectively. qPCR was positive in 95% of patients (95 for ttr and 89 for Inv A) while, pre-enriched blood qPCR was positive in 99%. The highest sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy was for qPCR for pre-enriched blood (99%, 100%, 100%, 99.01%, 99% respectively).* ***Conclusion:*** *The study highlighted the high accuracy of qPCR for genes Inv A and ttr in diagnosis of S. typhi from blood.*

INTRODUCTION

S. enterica includes human restricted pathogens *Salmonella typhi (S. typhi*) and *Salmonella paratyphi (S. paratyphi) A, B, C* and is associated with development of typhoid fever. It is a severe systemic infection transmitted as water borne, food borne and by person to person contacts via contaminated hands. In high income countries, the improvement in food and water sanitation has almost eradicated this infection. Nevertheless, in less developed regions in Asia and Africa, this infection is endemic where about 20 million cases and 200,000 deaths are encountered every year [1].

The classical clinical condition for diagnosis of typhoid fever is the step ladder increase in fever and toxicity [2]. These signs can be masked by inappropriate antibiotics use. Therefore, there is a need for appropriate laboratory diagnosis of typhoid fever with atypical sign to differentiate it from other febrile conditions [3]. The standard microbiological investigation for enteric fever is bacterial culture from bone marrow aspiration or a combination of blood, stool or urine cultures [4].

The bone marrow culture has good sensitivity above 90% with less implication to inhibition by vious antibiotic therapy. However, this method is invasive and not suitable for routine use [5]. The routine diagnosis relies upon blood culture and Widal test. However, both laboratory tests lack sensitivity and specificity [6-7].

The establishment of molecular methods such as polymerase chain reaction (PCR) provide an attractive method for detection of *S. enterica* as it is rapid method, sensitive, specific and it is unaffected by antibiotics intake. Various genes in *S. enterica* were used as target regions for detection by PCR such as genes coding somatic (O), flagellar (H), and Vi antigens of *S. Typhi* [8-10], as well as *Salmonella* pathogenicity islands (SPIs) [11], 16s RNA gene[12], or other gene complexes might be

essential for the invasion of *salmonella* into intestinal cells [13].

The pathogenesis of systemic *Salmonella* infection is related to the presence of complex virulence factors. These factors play an important role in the pathogenicity of *S. enterica* as an intracellular human pathogen and act through different stages of invasion, intracellular multiplication and survival within the host. The virulence genes of the *Salmonella* are found on large area of the genes of 10–135 kb named SPIs [14]. The SPIs are identified by the unique difference in the guanine and cytosine content from the core genome and are usually linked to specific tRNA genes and mobile genetic elements, as insertion sequence, transposons or bacteriophage genes [15].

Recent study has reported good sensitivity and specificity for qPCR method in detection of *Inv A* and *ttr* genes for *Salmonella* in stool and enriched broth media[16]. The *inv A* gene is found in the *Salmonella* pathogenicity island 1 that helps the pathogen to internalize into the human epithelial cells[17]. The *ttr* gene is present within *Salmonella* pathogenicity island 2 and codes for the tetrathionate reductase involved in the life cycle of *Salmonella*[18].

Previous studies had evaluated pre-enriched blood culture to be valuable strategy in increasing the sensitivity of PCR method for diagnosis of *S. enterica*[19-20].

In Egypt, there are insufficient data about the function of molecular methods in diagnosis of enteric fever. There is one report described good sensitivity for detecting Histidine receptor binding protein (his J) of *S. enterica* by qualitative PCR [21].

The aim of the current study was to evaluate the qPCR for detection of *Inv A* and *ttr* genes of *S. enterica* in blood specimens and pre-enriched blood in patients with typhoid fever.

METHODOLOGY

This was a case-control laboratory-based study that included one hundred patients with clinically diagnosed typhoid fever. In addition, one hundred control subjects with negative blood cultures were included to confirm the specificity of qPCR. The patients were recruited from Mansoura University Hospital from March 2021 till May 2022. The inclusion criteria were adult patients complaining of fever >38°C with ladder step character in the previous 72 hours along with gastrointestinal symptoms (abdominal pain, diarrhea, or constipation) and with no intake of antibiotics within the last week [2].

Ethical approval:

The study was approved by Mansoura Faculty of Medicine Ethical Committee (R.22.08.1794) and written approval was obtained from each participant.

Blood sample:

Ten milliliters of blood were obtained under sterile condition from each participant. 7ml of blood were inoculated to BacT/Alert blood culture bottle (biomerieux, USA) and promptly loaded into the instrument. 1ml was added to plain tube to separate serum for Widal test (Omega, UK). The remaining 2 ml blood were subdivided; 1ml kept untreated and 1 ml was pre-enriched immediately on equivalent volume of TSB with 5% bile (biomerieux, USA) in a BD Falcon™ conical tube (Becton Dickinson and Company, USA) and then incubated for 5 hours at 37°C. The pre-enriched blood and the untreated one were kept at -20°C for further study by qPCR [20].

Culture of Blood for *S. enterica*:

Positive blood culture was subjected for subculture on blood agar and MacConkey at 37C for one day. The growth of *Salmonella* was identified by Gram stain followed by API 20E (biomerieux, USA) according to the manufacturer's instructions. Serological testing for typing of *Salmonella* species was performed in accordance to Kauffmann-White classification [22] by using Remel agglutinating sera (ThermoFisher, USA) according to manufacturer's protocol.

Real- time PCR for detection of *Inv A* and *ttr* genes:

DNA extraction

DNA was extracted from blood samples and TSB bile enriched blood by using QIAamp DNA extraction kit (Qiagen, Germany) as the manufacturer's protocol. The extracted DNA was kept frozen at -20°C till time of qPCR.

Real -time PCR protocol

The preparation of 25 µl reaction mixture included adding 12.5 µl Platinum ™ qPCR Super Mix-UDG (Invitrogen, USA), 0.1 µl for each of forward and reverse primer for each gene, 0.1 µl specific probe at concentration of 200 nM for primers and probes, 0.05 µl ROX reference dye (Life Technologies, USA) at 50 nM final concentration, 5 µl of test DNA and 7.15 µl nuclease free water. The final mix was loaded into 96 well PCR plate. Five µl of extracted DNA from pure *Salmonella* culture were used as positive control and five µl of sterile distilled water were used as negative control. The qPCR procedure included initial denaturation at 95°C for one minute, followed by forty cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds using Applied Biosystem Real-Time PCR system (ThermoFisher, USA). The fluorescence was measured using the software of the qPCR instrument. Specimens with Cycle threshold (Ct) values of <35 cycles were considered positive. The used sequences of the primers and the probes of *Inv A* and *ttr* genes were listed in table 1 [16].

Table 1: The sequences of the used primers and probes

Gene	Primer sequence and Probe description
Inv A	**F:** 5′-AGCGTACTGGAAAGGGAAAG-3′ **R:** 5′-CACCGAAATACCGCCAATAAAG-3′ **Probe** FAM-TTACGGTTCCTTTGACGGTGCGAT-BHQ1
ttr	**F:** 5′-CTCACCAGGAGATTACAACATGG-3’ **R:** 5′-AGCTCAGACCAAAAGTGACCATC-3′ **Probe** FAM-CACCGACGGCGAGACCGACTTT-BHQ1

FAM: Fluorescein amidite; BHQ1: Black Hole Quencher-1

Statistical Analysis:

The data were interpreted by the use of SPSS 22. The numerical data were expressed as mean and standard deviation and the qualitative data were listed as number and percentages. The comparing of qualitative data was performed by chi-square test and the P value was significant if it was <0.05. The sensitivity, specificity, accuracy, PPV and NPV were estimated by the medcalc web-based application (https://www.medcalc.org/calc/diagnostic_test.php).

RESULTS

The study included 100 patients diagnosed clinically as typhoid fever by clinical examination and ladder step fever. The patients were 51 males and 49 females with mean age ± SD 45.11±13.68 years. They were mainly from rural residence (68%) with fever accompanied by diarrhea (64%) and fever accompanied with abdominal pain (38%), as presented in table 2. In addition, 100 control subjects were included, which were 62 males and 38 females with mean age ± SD 41.63±12.39 years.

Table 2: Demographic and clinical data of the studied patients (No.=100).

Age (mean± SD)	45.11± 13.68	
Sex		
Male (No. - %)	51	51%
Female (No. - %)	49	49%
Residence		
Rural (No. - %)	68	68%
Urban (No. - %)	32	32%
Abdominal pain (No. - %)	38	38%
Diarrhea (No. - %)	64	64%

Blood culture was positive for *S. enterica* in 51% of the patients and Widal test was positive with titer above 1/80 for antibodies to O antigen in 70% of the patients. Combined Widal and/or blood culture were positive in 82%. Blood qPCR for the studied genes was positive in 95% of the patients (95 for *ttr* gene and 89 for *Inv A* gene) and pre-enriched blood qPCR was positive in 99% of the patients (99 for *ttr* gene and 95 for *Inv A* gene). In the control subjects, all laboratory tests for *Salmonella* were negative except Widal test was positive in 11% of the control subjects (table 3).

Table 3: Microbiological laboratory findings of the studied subjects

laboratory findings	Patients (n=100)		Control (n=100)	
	No.	%	No.	%
Positive blood culture	51	51%	0	0%
Positive Widal test with antibody to O antigen>1/80	70	70%	11	11%
Combined Widal and/or blood culture	82	82%	11	11%
Positive real time PCR for blood	95	95%	0	0%
Inv A gene	89	89%		
ttr gene	95	95%		
Positive real time PCR for enriched blood	99	99%	0	0%
Inv A gene	95	95%		
ttr gene	99	99%		

The isolated *S. enterica* were 48 isolates *S. typhi* and three isolates were *S. paratyphi A*, data not shown.

The comparison between different microbiological laboratory tests for diagnosing *S. enterica* revealed that all positive blood culture samples were positive by blood qPCR for *S. enterica* with significant association between both tests (OR 1.1, 95%CI 1.013-1.22, P=0.02). Moreover, pre-enriched blood qPCR had all

positive samples with blood qPCR in addition to more 4 positive samples with significant association between both methods (OR 0.04, 95% CI 0.015-0.11, P=0.001). Widal test revealed combined positive results with blood qPCR in 68 of the patients with two Widal positive samples had negative qPCR (OR 3.78, 95%CI 0.59-23.9, P=0.13). Combined positive Widal and/ or blood culture revealed 80 positive results with qPCR with more two positive results negative by blood qPCR with significant association between both tests (OR 8.00, 95% CI 1.23-52.02. P=0.012). (Table 4).

Table 4: Comparison between different microbiological laboratory methods for diagnosis of *S. enterica*

	Real time PCR for blood				P value	OR	95%CI
	Positive (n=95)		Negative (n=5)				
	No.	%	No.	%			
Blood culture					0.02*	1.1	1.013-1.22
• Positive	51	53.7%	0	0%			
• Negative	44	46.3%	5	100%			
Pre-enriched blood real time PCR							
• Positive	95	100%	4	80%	0.001*	0.04	
• Negative	0	0%	1	20%			.0015-0.11
Widal test							
• Positive	68	71.6%	2	40%	0.13	3.78	0.59-23.9
• Negative	27	28.4%	3	60%			
Combined Widal and/or blood culture							
• Positive	80	84.2%	2	40%	0.012*	8.0	1.23-52.02
• Negative	15	15.8%	3	60%			

** P value was significant if <0.05*

As demonstrated in table 5, the highest sensitivity, specificity, PPV, NPV and accuracy was for qPCR for pre-enriched blood samples (99%, 100%, 100%, 99.01%, 99% respectively) followed by qPCR for blood samples (95%, 100%, 100%, 95.2%, 97.5% respectively). The lowest sensitive was blood culture (51%) with specificity 100% and accuracy 75.5%. Widal test sensitivity, specificity, PPV, NPV and accuracy were 70%, 89%, 86.4%, 74.8% and 79.5% respectively. The combined Widal and/or blood culture results increased the sensitivity, specificity, PPV, NPV and accuracy to 82%, 89.2%, 88.2%, 83.2% and 85.5% respectively.

Table 5: Sensitivity, specificity, PPV, NPV and accuracy of microbiological laboratory tests for detection of *S. enterica*.

laboratory findings	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
Blood culture	51%	100%	100%	67.11%	75.5%
Real time PCR for blood	95%	100%	100%	95.2%	97.5%
Real time PCR for pre-enriched blood	99%	100%	100%	99.01%	99%
Widal test	70%	89%	86.4%	74.8%	79.5%
Combined Widal and/or blood culture	82%	89.2%	88.2%	83.2%	85.5%

DISCUSSION

Salmonella enterica is the cause of typhoid fever, a health issue in less developed countries. The typical clinical diagnosis depends upon fever associated with other symptoms such as abdominal pain and diarrhea [23-24].

In this study, the typhoid fever was diagnosed clinically by step ladder fever accompanied by diarrhea (64%) and abdominal pain (38%). In another study from Mansoura University hospital, fever was the predominant sign with diarrhea in 54% and abdominal pain in 52.2% of the patients with typhoid [21]. These symptoms are common in *S. enterica* infection [25- 26]. The majority of our patients (68%) were from the rural residence. This finding can be attributed to the poor hygiene and inadequate water sanitation conditions in these areas [27].

The definitive laboratory diagnosis of enteric fever is the microbiological culture of body fluids such as

blood culture and bone marrow culture [27-28]. In our study, blood culture for *S. enterica* was positive in 51%. The sensitivity was 51%, specificity was 100% and accuracy was 75.5%.

In the same context, the sensitivity of blood culture ranges from 40% up to 75% in previous studies [29- 30]. The low sensitivity of blood culture for diagnosing *S. enterica* is attributed to effects of antibiotics therapy and subside of bacteremia in later stages of infection [30- 31]. There is a decline in the positivity of blood culture from 90% in the 1st week up to 50% in the 3rd week of untreated infected patients [21] leading to difficulty to depend upon blood culture alone as a principal diagnostic test in enteric fever.

The total isolated *S. typhi* by blood culture were 48 isolates and isolated *S, paratyphi A* were 3 isolates in our study. Similarly, previous reports revealed the *S. typhi* as a predominant etiology of enteric fever, followed by *S. paratyphi A*[32- 33].

Widal test is a serological test used for diagnosis of typhoid with some limitations including establishment of base line titer for different population, cross reactivity with other pathogens and the need for repeated samples 10 to 14 days apart to confirm positivity [34- 35].

In the current study, Widal test with antibody titer for O antigen >1/80 was detected in 70% of the patients and in 11% of the control subjects. Widal test sensitivity, specificity, PPV, NPV and accuracy were 70%, 89%, 86.4%, 74.8% and 79.5% respectively. Similar results were reported in previous systemic review reported the mean sensitivity, specificity, PPV and NPV of 73.5 with SD of 12.6 minimum of 45.2 and maximum of 98, 75.7 with SD of 20.2 minimum of 13.8 and maximum of 98, 75.2 with SD of 24.8 minimum of 31 and maximum of 100 and 60 with SD of 29 minimum of 5.7 and maximum of 91 respectively [36]. Moreover, Mawazo et al. [37] from Tanzania revealed similar results for Widal testing.

The lack of sensitivity and specificity of Widal test with the necessary to repeat the test apart within 10 to 14 days make it unsuitable for rapid diagnosis of enteric fever [36, 38]. The combined Widal and/or blood culture results in the current study increased their sensitivity, specificity and accuracy to 82%, 89.2% and 85.5% respectively. Actually, both tests are used in combination for diagnosing enteric fever in less developed regions as cheap, easy and available options

Nucleic acid amplification techniques for diagnosis of *S. enterica* in blood have been established and evaluated previously [39- 40].

In this study, monoplex qPCR was used for detecting *Inv A* gene and *ttr* gene in blood samples. The test is rapid than culture even with the use of pre-enrichment step and can be used for batch processing of the specimens. The used primers and the probes for the *ttr* and *Inv A* were previously evaluated for use in stool samples and demonstrated the ability to evaluate various *Salmonella strains*, including *S. Enteritidis*, *S. Typhimurium* and *S. Typhi* [16].

In our study, the highest sensitivity, specificity, PPV, NPV and accuracy was for qPCR for pre-enriched blood samples (99%, 100%, 100%, 99.01%, 99% respectively) followed by qPCR for blood samples (95%, 100%, 100%, 95.2%, 97.5% respectively). Chirambo et al. [16] reported the sensitivity of monoplex qPCR for these genes ranged from 90.3% to 99.53% and the specificity ranged from 90.31% to 99.3% according to the used genes.

The adding of pre-enrichment step for blood sample in the current study, increases the sensitivity and specificity of qPCR to 99%, 100% with accuracy 99%. The increase yield of qPCR after adding bile to enrichment media is attributed to the release of bacteria from intracellular compartment of the blood sample which leads to increase two folds in bacterial numbers, in addition to dilution of serum bactericidal effect [20].

CONCLUSION

The present study highlights the good accuracy of the qPCR for genes *Inv A* and *ttr* in diagnosis of *S. typhi* and *S. paratyphi A* from blood samples. The short pre-enrichment step of the blood specimen by media containing bile increases the sensitivity of the qPCR.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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