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Validation Of Polymerase Chain Reaction For Diagnosis Of Typhoid Fever In Egyptian Patients

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Abstract:

Background: Typhoid fever continues to be a major health problem in many parts of the world, particularly the developing countries. The rate of typhoid fever is increasing in Egypt. Clinical diagnosis of enteric fever is difficult, as the symptoms associated with enteric fever overlap with those of other febrile illness. The objective of this study is to validate a PCR technique for the detection of typhoid infection. So, we can differentiate between the true and current infections by this organism from the falsely positive antibodies produced from previous or treated infections.

Methods: This study comprised 115 patients (88 males and 27 females) from different ages experienced the clinical symptoms of typhoid fever. This study was carried out at Mansoura University Hospitals. The blood samples collected were subjected to culture, Widal test and PCR targeting the hto gene of S. Typhi.

Results: PCR standardized for hisJ (hto) gene result in specific amplicon of 495-bp. In positive PCR cases of typhoid (total 15), a single band of 495bp corresponding to hisJ (hto) gene was obtained. The PCR assay was found to be highly specific and the true positive typhoid cases (n = 9) are positive of Widal test, blood culture and/or PCR. PCR was sensitive than blood culture for diagnosis of typhoid fever.

Conclusion: We recommend using PCR as an alternative to blood culture for diagnosis of typhoid fever. We also recommend performance of a large scale study to test this PCR technique for screening for tyhoid fever in Egypt.

Key words : Typhoid fever, widal test, blood culture, PCR.

Introduction

Salmonella is an important enteric pathogen and is involved in causing both systemic and intestinal diseases in humans and a wide range of other hosts and food poisoning worldwide [3]. Serotypes within subspecies I (Salmonella enterica subsp. enterica) are responsible for the vast majority of salmonellosis in warm blooded animals. S. enterica subsp. enterica serovar Typhi and Paratyphi A, B and C are responsible for causing typhoid and paratyphoid fever in humans, with no age exemption, but it is less common in children younger than 2 years.

Typhoid and paratyphoid fever (also collectively called as enteric fever) continues to be a major health problem in many parts of the world particularly the developing countries. Infection occurs when water or food contaminated with serovar Typhi or Paratyphi A, B and C is consumed. These gram-negative rod-shaped bacteria are pathogenic only in humans and can be cultured from blood and stool of infected patients. Most patients who recover from the infection are able to eliminate the bacterium completely from their bodies. However, some of them may remain as healthy carriers, continuously shedding viable organisms in their stools posing severe threat of spread of infection to the susceptible population [2].

According to one estimate, the worldwide incidence of typhoid fever is between 13.5–26.9 million cases annually [5]. The problem of typhoid fever has been exacerbated by the appearance of multiple drug resistant strains [1], the treatment of which would depend on newer and advanced antibiotics as well as early and specific diagnosis. Therefore, there is immense need to get equipped with sensitive and specific assays for rapid detection and differentiation of Salmonella serovars responsible for enteric fever in human.

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Clinical diagnosis of enteric fever is difficult, as the symptoms associated with enteric fever are not unique and overlap with those of other febrile illness. Therefore, confirmation requires laboratory testing. Conventional methods used for laboratory diagnosis include culture, biochemical and serological testing which are time consuming (5-7 days), costly and technically demanding.

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. Widal test is quite sensitive but has become highly nonspecific. 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. 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% [4].

Blood culture are universally practiced in diagnostic procedures, because other methods are either invasive, or have failed to prove their utility or they are expensive, but Molecular diagnosis techniques target the pathogen itself so they are useful in early detection of disease. PCR is the gold standard method used for the diagnosis of a number of infectious diseases .The sensitivity and specificity of different cut-off titers of Widal test have also been evaluated using PCR as Gold standard test [6].

Polymerase chain reaction (PCR) has successfully been used to detect pathogens from a variety of clinical, environment as well as food samples and is an excellent tool for the early diagnosis of typhoid. In typhoid fever it can be an effective tool because it can be used even in cases where antibiotic therapy has been started or the pathogen load is very low [2].

Histidine receptor binding protein (hisJ) can be used for simultaneous detection and differentiation of Typhi and Paratyphi A from other salmonellae [2].

PCR reaction, Oligonucleotide primers of the histidine transport operon of Salmonella typhimurium was selected because it was considered to be highly conserved among species of Salmonella [7].

The aim of this study is to validate a PCR technique for the detection of typhoid infection. So, we can differentiate between the true and current infections by this organism from the falsely positive antibodies produced from previous or treated infections.

For typhoid fever patients, oligonucleotide primers of 25 bp defined the amplified region of a 496-bp segment of the histidine transport operon of Salmonella typhimurium (hto gene). This gene was selected because it was considered to be highly conserved among species of Salmonella [17, 18].

Subjects and methods

This study comprised 115 patients (88 males and 27 females) from different ages (Mean ± SD; 43.61 ± 10.87) experienced the clinical symptoms of typhoid fever. This study was carried out at Mansoura University Hospitals in the period between January 2019 and September 2020. This study was approved by the local ethics committee, and a written consent was taken from patients.

All patients with typhoid fever were subjected to: complete history taking with special emphasis on clinical symptoms of typhoid fever; including fever, vomiting and diarrhea, abdominal pain, and constipation. Moreover, Widal test was performed as a routine test for diagnosis of the typhoid fever. The inclusion criteria included adult patients with Widal titre (O antigen) 1/80, 1/160, and 1/320. Meanwhile, the exclusion criteria included Widal titre (O antigen) less than 1/80. Participants were already on antibiotic treatment and those who were diagnosed for other known febrile illness were excluded from the study.

Total leukocytic count, hemoglobin (Hb %), and platelets count were measured in all the studied patients with typhoid fever using an electronic cell counter (D-Cell 60 Hematology Analyzer, DIAGON Kft., Hungary). The serum AST, ALT, albumin, bilirubin, creatinine, and fasting blood glucose were measured in all the studied patients with typhoid fever using BT3500 automated chemistry analyzer (Biotecnica Instruments,S.p.A. via Licenza, 18 00156 Rome, Lazio, Italy).

For patients with typhoid fever, qualitative slide agglutination was performed using febrile antigen kits of Salmonella typhi (Chromatest Febrile Antigens kits, LINEAR CHEMICALS, S.L.U. Joaquim Costa 18 2ª planta. 08390 Montgat (Barcelona) SPAIN Telf. (+34) 934 694 990; E-mail: info@linear.es ; website: www.linear.es NIF-VAT:B60485687). The slide agglutination test was used as a screening test for the presence of anti TO and anti TH antibodies in the patient's serum. For the slide agglutination test, a drop of Salmonella typhi O and H commercial antigens are added on a drop of serum on test card and rotated at 100 rpm for one minute on an orbital shaker. Positive Widal test (O antigen) is determined by the formation of visible agglutination reaction from the mixture of S. enterica

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serotype Typhi antigen with the patient's serum antibody on the testing card. Results are reported as a semiquantitative test. Positive samples were diluted to know the Widal titre (O antigen) [8, 9, 10].

All samples were collected in BacT/ALERT bottles (BIOMERIEUX, USA) irrespective of antibiotics administration. Blood samples were subjected to automated blood culture system. Specific identification of all culture positive samples was accomplished by sub-culture on Blood agar, Chocolate agar and MacConkeys agar media (OXOID CO. UK). Inoculated Blood agar and MacConkeys agar plates were incubated aerobically at 37 ^{o}C. The Chocolate agar plates were incubated at 37 ^{o}C under 5-10% CO_2 condition (Candle jar) and examined after 18-24 hours of incubation. Identification of growth was performed by VITEK 2 Compact system (bioMérieux,France) [11, 12, 13, 14, 15].

Extraction of DNA was done using the QIAamp DNA blood Kit (Qiagen) following the instructions published by the manufacturer [16]. The cells (maximum 2 x 109 cells) were harvested in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm), and then supernatant was discarded. The pellet was resuspended in 180 µl Buffer ATL. The proteinase K (20 µl) was added and was mixed thoroughly by vortexing, and then was incubated at 56°C, and then was placed in a thermomixer to disperse the samples. Buffer AL (200 µl) was added to the sample, and mix thoroughly by vortexing. Then 200 µl of ethanol (96–100%) was added, and mixed again thoroughly by vortexing. The mixture was pipetted into the DNeasy Mini spin column which was placed in a 2 ml collection tube, and then was centrifuged at ≥ 6000 x g (8000 rpm) for 1 min. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), then 500 µl Buffer AW1 was added and centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute. The DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), and then buffer AW2 (500 µl) was added, and was centrifuged for 3 minutes at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a clean 2 ml microcentrifuge tube, and 200 µl Buffer AE was pipetted directly onto the DNeasy membrane and finally was incubated at room temperature for 1 min, and then was centrifuged for 1 min at ≥ 6000 x g (8000 rpm) to elute. For maximum DNA yield, the elution step was repeated once again.

For typhoid fever patients, oligonucleotide primers of 25 bp defined the amplified region of a 495-bp segment of the histidine transport operon of Salmonella typhimurium (hto gene). This gene was selected because it was considered to be highly conserved among species of Salmonella. The PCR primers were designed using a software program. The primer sequences for the forward and reverse oligonucleotides, from 5' to 3', were as follows: forward strand, ACTGGCGTTATCCCTTTCTCTGGTG; reverse strand, ATGTTGT- CCTGCCCCTGGTAAGAGA [17, 18].

The initial part of PCR optimisation was performed using conventional PCR. Each reaction in a total of 25 µL contained 12.5 µL PCR Master Mix (Promega, USA), 1 µL each of the forward and reverse primers, 5 µL of DNA template and 5.5 µL molecular grade water. The primer concentration used in the reaction was kept constant at 10 µM. Amplification was performed in Stepone thermal cycler. Annealing temperature optimisation was performed at 12 points using the following condition; initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C denaturation for 1 min, annealing at 49.8 °C to 65.1 °C for 1 min and extension, 72 °C for 1 min [19, 20]. PCR products (5 µL) were electrophoresed on 2% agarose gel in a 0.5× TBE buffer at 85 V for 60 min. The gel was stained with GelRed™ (Biotium®, USA). A 50 bp DNA ladder (Fermentas, USA) was included as the molecular weight marker in every electrophoresis run. The PCR products were visualised under ultraviolet (UV) illumination using gel image documentation system (GelDoc 1000 system, Bio-Rad, USA).

Statistical Analysis

Data analysis will be performed using statistical software program (SPSS for Windows, version 21, USA). All the nominal data, regarding clinical presentation and demographic data, as well as Widal titre and brucella titre results were expressed as number (percentage). Chi-square test will be used to assess the distribution of such nominal data between the selected patients. However, for the numerical data, descriptive statistic, including mean ± standard deviation, Median (Range), and 95 % Confidence interval of mean (95 % CI), was presented. Association between Widal titre and other diagnostic methods; blood culture and PCR in patients with typhoid as well as the association between widal titre and clinical symptoms in patients with typhoid fever was analyzed using Chi-Square test. Moreover, the association between bucella titre and other diagnostic methods; blood culture and PCR in patients with brucellosis as well as the association between brucella titre and clinical symptoms in patients with brucellosis was analyzed using Chi-Square test. Correlation between different blood parameters in patients with typhoid fever and in patients with brucellosis was assessed using Pearson

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correlation. Finally, ROC curve was done to assess the sensitivity and specificity of blood culture and PCR as a diagnostic method for typhoid fever and brucellosis. For all statistical examinations, results were considered significant at P-value ≤ 0.05.

Results

Table 1. Demographic Data of patients with Typhoid fever

Sex		Chi-square = 32.357 P-value = 0.001
Males No (%)	88 (76.5 %)	
Females No (%)	27 (23.5 %)	
Age (Years)		Chi-square = 41.125 P-value = 0.378
Male (Mean ± SD)	43.61 ± 10.25	
Female (Mean ± SD)	41.63 ± 10.87	
Widal titre		Chi-square = 92.887 P-value = 0.001
1/80 No (%)	87 (75.7 %)	
1/160 No (%)	12 (10.4 %)	
1/320 No (%)	16 (13.9 %)	

Table 1 summarizes the demographic data of patients with Typhoid fever. Males were 88 (76.5 %) with ages (Mean ± SD) 43.61 ± 10.25 and Females were 27 (23.5 %) with ages (Mean ± SD) 41.63 ± 10.87. For widal titre, the highest percentage was for titre 1/80. P-values for chi-square test were significant in both sex and widal titre.

Table 2. Widal titre in patients with Typhoid fever

	Widal titre			Chi-square
	1/80 (n = 87)	1/160 (n = 12)	1/320 (n = 16)	
Sex				Chi-square = 0.683
Male (n = 88)	68 (77.3 %)	9 (10.2 %)	11 (12.5 %)	P-value = 0.711
Female (n = 27)	19 (70.4 %)	3 (11.1 %)	5 (18.5 %)	
Age	43.47 ± 9.96	39.83 ± 11.31	43.88 ± 12.10	Chi-square = 85.133 P-value = 0.272

Table 2 summarizes the Widal titre in patients with Typhoid fever. There was a non-significant (p = 0.711) association between widal titre and both sex and age.

Table 3. Distribution of clinical symptoms in patients with typhoid fever

	Present	Not present	Total
Fever	74 (64.3 %)	41 (35.7 %)	115 (100 %)
Vomiting and diarrhea	63 (54.8 %)	52 (45.2 %)	115 (100 %)
Abdominal pain	60 (52.2 %)	55 (47.8 %)	115 (100 %)
Constipation	18 (15.7 %)	97 (84.3 %)	115 (100 %)

Table 3 summarize Distribution of clinical symptoms in patients with typhoid fever. The recorded clinical symptoms were fever in 74 (64.3 %) of patients, vomiting and diarrhea in 63 (54.8 %) of patients, abdominal pain in 60 (52.2 %) of patients, and constipation in 18 (15.7 %) of patients.

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Table 4. TLC, Hemoglobin, and Platelets count in patients with Typhoid Fever (n = 115)

	Mean ± SD	Median (Range)	95% CI of Mean
TLC (X 10^3)	5.875 ± 0.106	5.6 (4.2 – 7.5)	5.664 – 6.085
Hemoglobin (gm/dL)	14.18 ± 1.03	14.0 (12.9 – 16.0)	13.99 – 14.37
Platelets (X 10^3)	237.9 ± 49.4	231 (144 – 310)	228.7 – 247

Table 4 summarizes the descriptive statistics for TLC, hemoglobin, and platelets count in patients with Typhoid Fever. Total leukocyte count (X 10^3) was 5.875 ± 0.106, Hemoglobin (gm/dL) percent was 14.18 ± 1.03, and platelets count (X 10^3) was 237.9 ± 49.4.

Table 5. AST, ALT, Albumin, bilirubin, creatinine, and Fibrinogen levels in patients with Typhoid Fever

	Mean ± SD	Median (Range)	95% CI of Mean
AST (U/L)	33.83 ± 7.35	31 (21 – 56)	30.99 – 35.67
ALT (U/L)	34.57 ± 5.79	30 (22 – 55)	31.65 – 36.50
Albumin (gm/dL)	4.43 ± 0.61	4.50 (3.30 – 5.80)	4.32 – 4.54
Bilirubin (mg/dL)	0.82 ± 0.16	0.80 (0.50 – 1.10)	0.79 – 0.85
Creatinine (mg/dL)	0.91 ± 0.11	0.90 (0.80 – 1.30)	0.89 – 0.93
Fasting blood glucose (gm/dl)	95.84 ± 12.1	98 (70 – 130)	93.61 – 98.08

Table 5 summarizes the descriptive statistics for AST, ALT, albumin, bilirubin, creatinine, and fibrinogen levels in patients with Typhoid Fever. AST (U/L) level was 33.83 ± 7.35, ALT (U/L) was 34.57 ± 5.79, albumin (gm/dL) level was 4.43 ± 0.61, bilirubin (mg/dL) level was 0.82 ± 0.16, creatinine (mg/dL) level was 0.91 ± 0.11, and fasting blood glucose (gm/dl) level was 95.84 ±

Table 6. Correlation of different blood parameters in patients with typhoid fever

	TLC	Hemoglobin	Platelets	AST	ALT	Albumin	Bilirubin	Creatinine	FBG	Widal titre
TLC		r = 0.111 p = 0.237	r = 0.020 p = 0.830	r = 0.031 p = 0.740	r = -0.060 p = 0.526	r = -0.083 p = 0.379	r = 0.003 p = 0.973	r = -0.157 p = 0.095	r = -0.019 p = 0.839	r = -0.141 p = 0.134
Hemoglobin			r = 0.087 p = 0.355	r = -0.084 p = 0.372	r = -0.090 p = 0.338	r = 0.001 p = 0.993	r = 0.089 p = 0.346	r = -0.165 p = 0.078	r = -0.014 p = 0.879	r = -0.105 p = 0.266
Platelets				r = 0.219 * p = 0.019	r = 0.221 * p = 0.018	r = -0.051 p = 0.586	r = -0.133 p = 0.155	r = -0.127 p = 0.178	r = 0.048 p = 0.608	r = 0.085 p = 0.365
AST					r = 0.719 ** p = 0.001	r = -0.013 p = 0.892	r = 0.163 p = 0.082	r = -0.037 p = 0.694	r = -0.064 p = 0.494	r = 0.062 p = 0.513
ALT						r = 0.088 p = 0.347	r = -0.049 p = 0.606	r = -0.202 * p = 0.031	r = 0.021 p = 0.827	r = 0.131 p = 0.164
Albumin							r = -0.148 p = 0.115	r = -0.038 p = 0.690	r = -0.002 p = 0.980	r = -0.011 p = 0.905
Bilirubin								r = 0.176 p = 0.060	r = -0.064 p = 0.496	r = -0.097 p = 0.300
Creatinine									r = 0.024 p = 0.801	r = 0.028 p = 0.769
FBG										r = -0.119 p = 0.205
Widal titre										

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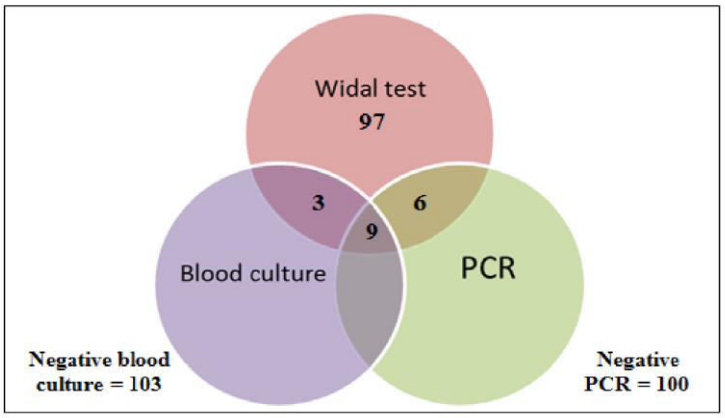

Figure 1. Venn diagram showing number of positive results in each combination of Widal test, blood culture and PCR (n = 115). The true positive typhoid cases (n = 9) are positive of Widal test, blood culture and/or PCR

Table 7. Association between Widal titre and other diagnostic methods; blood culture and PCR in patients with typhoid

	Blood culture		PCR	
	Negative	Positive	Negative	Positive
Widal titre				
1/80	83 (80.6 %)	4 (33.3 %)	85 (85.0%)	2 (13.3 %)
1/160	9 (8.7 %)	3 (25.0 %)	8 (8.0 %)	4 (26.7 %)
1/320	11 (10.7%)	5 (41.7 %)	7 (7.0 %)	9 (60.0 %)
Total	103 (100 %)	12 (100 %)	100 (100 %)	15 (100 %)
	Chi-Square value = 13.313 P-value = 0.001		Chi-Square value = 39.545 P-value = 0.001	

Table 7 summarizes the correlation between Widal titre and other diagnostic methods; blood culture and PCR in patients with typhoid. There was a significant association between widal titre and blood culture ($p = 0.001$) in patients with typhoid fever. Furthermore, there was a significant association between widal titre and PCR ($p = 0.001$) in patients with typhoid fever.

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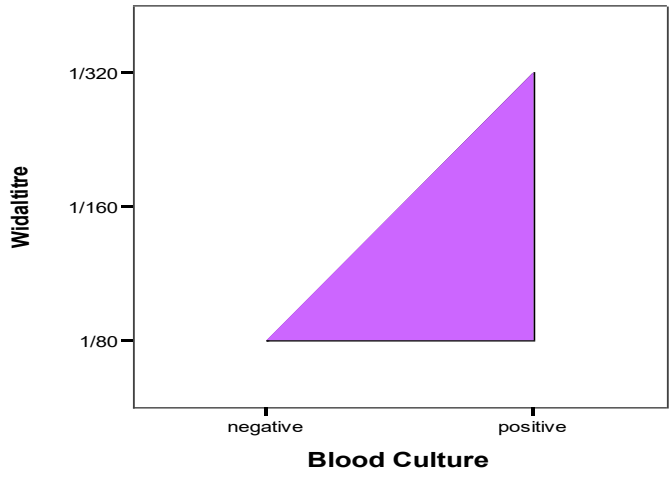

Figure 2. Correlation between Widal titre and blood culture in patients with typhoid

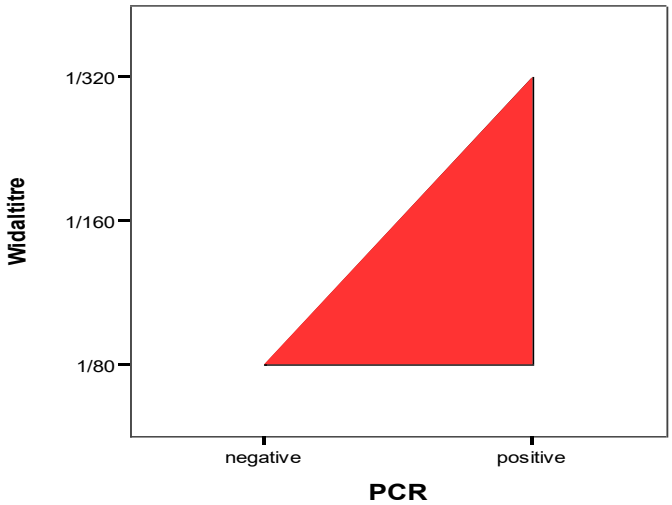

Figure 3. Correlation between Widal titre and PCR in patients with typhoid

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Table 8. Association between widal titre and clinical symptoms in patients with typhoid fever

	Widal titre			Total	Chi-square Test
	1/80	1/160	1/320		
Fever					
Negative	35 (85.4 %)	3 (7.3 %)	3 (7.3 %)	41 (100 %)	Chi-Square value = 3.381
Positive	52 (70.3 %)	9 (12.2 %)	13 (17.6 %)	74 (100 %)	P-value = 0.184
Vomiting and diarrhea					
Negative	44 (84.3 %)	4 (7.7 %)	4 (7.7 %)	52 (100 %)	Chi-Square value = 4.332
Positive	43 (68.3 %)	8 (12.7 %)	12 (19 %)	63 (100 %)	P-value = 0.115
Abdominal pain					
Negative	43 (78.2 %)	5 (9.1 %)	7 (12.7 %)	55 (100 %)	Chi-Square value = 0.378
Positive	44 (73.3 %)	7 (11.7 %)	9 (15 %)	60 (100 %)	P-value = 0.828
Constipation					
Negative	73 (75.3 %)	10 (10.3 %)	14 (14.4 %)	97 (100 %)	Chi-Square value = 0.143
Positive	14 (77.8 %)	2 (11.1 %)	2 (11.1 %)	18 (100 %)	P-value = 0.931

Table 8 explains the correlation between widal titre and clinical symptoms in patients with typhoid fever. There was a non- significant association between widal titre and clinical symptoms, including fever, vomiting and diarrhea, abdominal pain, and constipation in patients with typhoid fever.

Table 9. The validity of Blood culture, and PCR test as a diagnostic tool for Typhoid fever

	Blood culture	PCR
AUC	0.010	0.005
Sensitivity (%)	87.5	92.86
Specificity (%)	96.90	98.97
PPV (%)	82.03	93.66
NPV (%)	95.31	97.01
Accuracy (%)	93.04	96.52
Standard error	0.008	0.006
CI 95 %	0.006 – 0.026	0.006 – 0.016
P - value	0.001	0.001

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Table 9 showed the validity of Blood culture, and PCR test as a diagnostic tool for Typhoid fever. PCR was sensitive than blood culture for diagnosis of typhoid fever.

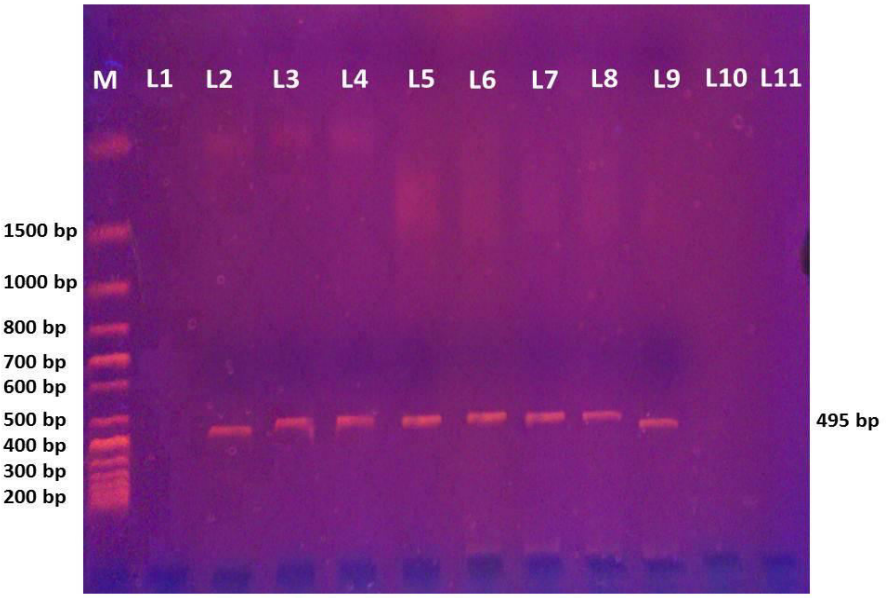

Figure 4. Agarose gel electrophoresis of PCR amplicons after amplification of gene-specific DNA fragments from whole blood sample for patients with typhoid fever. Samples were analyzed by electrophoresis through 3% (wtvol) agarose gels. Gels were stained with ethidium bromide and DNA was visualized under UV. This results in amplicons 495 bp.

Discussion

Typhoid fever is widely recognized as a major public health problem in most developing tropical countries [30]. It is a systemic infectious disease characterized by an acute illness; the first typical symptoms are fever, headache, abdominal pain etc. Human being is the only reservoir and host for typhoid fever and is transmitted by faecally contaminated water and food in endemic areas especially by carriers handling food [31]. These diseases have been associated with major negative economic impact in regions where it is widely spread [32]. The liver is enlarged during the second week of infection of typhoid fever where the bacteria cause the inflammation of the liver, leading to damage of the liver.

Gender wise in the current study, males were found to be more prominently affected than females. Males were 88 (76.5 %) and Females were 27 (23.5 %). This might be due to the fact that males are the earning members of the family spending most of the time out of home, and so making him liable to eat outside home [33]. In the present study, anti-TO titer of 1:80 was the highest reported titer in our study, an indicatior for typhoid fever infection.

In the current study, in patients with typhoid fever, the recorded clinical symptoms were fever in 74 (64.3 %) patients, vomiting and diarrhea in 63 (54.8 %) patients, abdominal pain in 60 (52.2 %) patients, and constipation in 18 (15.7 %) patients. Classically, the progression of untreated typhoid fever is divided into four distinct stages, each lasting about a week. Over the course of these stages, the patient becomes exhausted and emaciated. In the first week, the body temperature rises slowly, and fever fluctuations are seen with relative bradycardia , malaise, headache, cough, and abdominal pain. In the second week, the person is often too tired to get up, with high fever and bradycardia. Delirium can occur, where the patient is often calm. The abdomen is distended and painful in the right lower quadrant, where a rumbling sound can be heard. Diarrhea can occur in this stage, but constipation is also common. The spleen and liver are enlarged (hepatosplenomegaly) and tender. In the third week of typhoid fever, several complications can occur [34, 35].

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In the current study, in the patients with typhoid fever under study, the total leukocyte count was $5.875 \times 10^3 \pm 0.106$.

In other studies, a decrease in the number of circulating white blood cells (leukopenia) occurs with eosinopenia and relative lymphocytosis; blood cultures are positive for Salmonella enterica subsp. enterica serovar Typhi. The Widal test is usually negative in the first week. Leucopenia with neutropenia and a relative lymphocytosis are believed to be common findings in patients with typhoid fever [36, 37].

Abdool Gaffar et al., 1992, reviewed 191 adult patients with typhoid. The total and differential leucocyte counts done on admission were analysed. Leucopenia was found in only 24.6% of patients. Whilst complications occurred at any white cell count, the prevalence of complications increased significantly to 70% in patients with a white cell count above 8 x 10(9)/l. Neutropenia was found in 25% of patients, and none of the patients had an absolute lymphocytosis, whereas 75.8% of patients had true lymphopenia [36].

Dhillon et al., 2017, reported that 6.5% of their blood culture positive patients had leucopenia [38]. Kakaria et al., 2014, reported leucopenia in 10 % of their patients with typhoid fever [39]. Dheer et al., 2012, also reported leucopenia in 7.5% of typhoid fever patients [40]. Dhillon et al., 2017, reported that most of their cases had normal total leucocyte counts like earlier reports. Also, they reported that eosinopenia was seen in 6.5% of patients of definitive typhoid fever. It is seen that eosinopenia often absolute may be present in 70- 80% of cases. Presence of absolute eosinopenia offers a clue to diagnosis but does not differentiate it from other acute bacterial or viral infections. In their study eosinopenia is not a significant finding [38].

In the existing study, platelets count was $237.9 \times 10^3 \pm 49.4$.

Dhillon et al., 2017, reported that thrombocytopenia was seen in 21.7% of blood culture positive patients of typhoid fever in their study [38]. It is seen that overall prevalence of thrombocytopenia is around 10-15%. However, Iqbal et al., 2015, had seen isolated thrombocytopenia in 43% of cases typhoid fever in their study [41]. Shilpa et al., 2017, had shown thrombocytopenia in 17.24%of patients with enteric fever in their trial [42]. Shrivastava et al.,2015, observed Thrombocytopenia in 39.7%in their study [43]. Malik et al., 2001, found thrombocytopenia in 26% of typhoid fever cases in their trial [44].

In the current study, Hemoglobin (g/dL) percent was 14.18 ± 1.03. Hemoglobin is normal in the initial stages but drops with progressing illness. Kakaria et al., 2014, reported Anemia in 42.9% in their prospective study [39]. Shilpa et al., 2017, observed anemia in 34% of their enteric fever patients which is attributed to the maturational arrest of the cell lines in salmonella infection in bone marrow [42, 45]. Severe anemia is unusual in typhoid fever and should make one suspect intestinal hemorrhage or hemolysis or an alternative diagnosis like malaria.

In the current study, in patients with typhoid fever, Aspartate transaminase “AST” (U/L) level was 33.83 ± 7.35, Alanine aminotransferase “ALT” (U/L) was 34.57 ± 5.79, albumin (gm/dL) level was 4.43 ± 0.61, and bilirubin (mg/dL) level was 0.82 ± 0.16.

However, in previous studies, increased serum levels of the various enzymes in the liver function tests carried out on patients with typhoid fever only are indicative of a hepatocyte disorder. The liver function tests are used for evaluation of hepatic involvement during typhoid fever [46]. According to Ali et al., 2007, elevated serum enzymes (alkaline phosphatase “ALP”, Aspartate transaminase “AST” and Alanine aminotransferase “ALT”) in his work were discovered in 85% of patients with typhoid fever [47]. Morgenstern and Hayes (1991) and Mirsadraee et al. (2007) in their findings reported that 62 and 70%, respectively of patients with typhoid fever had elevated AST and ALT while Rasoolinejad et al., 2003, reported that 74% of patients with typhoid fever had elevated ALP [48, 49, 50].

For patients with malaria and typhoid co-infection, the results show a significant increase ($p<0.05$) in the levels of ALP, AST, ALT, TB and CB [46]. Kayode et al., 2011, reported elevated bilirubin (TB and CB) in malaria and in typhoid co-infection patients which are as a consequence of haemolysis, but in severe cases can lead to the damage of the liver [51]. For the increase in the levels of ALP, AST and ALT, Mbuh et al., 2003, reported that patients with malaria and typhoid co-infection have increased ALP, AST and ALT [52]. Kanjilal et al., 2006, in their work discovered that 87.6% of patient with malaria and typhoid co- infection had elevated ALP, AST and ALT [53].

In the current study, in patients with typhoid fever, creatinine (mg/dL) level was 0.91 ± 0.11.

In another study conducted by Kumar et al., (2005), the authors reported a significant increase in creatinine level of typhoid fever positive female compared to their control which may lead to Azotemia and increased levels of Nitrogenous compound in the blood [54].

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In the existing study, fasting blood glucose (gm/dl) level was 95.84 ± 12.1.

Blood glucose level is an essential part of our body. In earlier studies, serum glucose concentration was significantly lower in typhoid patients as compared to that in their normal healthy individuals. Hypoglycemia has rarely been described as a clinical sign of severe bacterial sepsis. Glucose level was significantly lower in typhoid patients as compared to normal individuals. S. typhi requires glycolysis and glucose for successful infection of macrophages that cause infection. By this glucose level is low in typhoid fever patients [55].

Enteric fever is still serious public health problem in many geographic areas and is endemic in Africa. The clinical syndrome caused by typhoid and paratyphoid fever overlaps with many other febrile illnesses. Typhi and Paratyphi serovars may sometimes cause fatal infections of adults and children that cause bacteremia and inflammatory destruction of the intestine and other organs that require urgent treatment by the administration of appropriate antibiotics.

Recently, multiple-drug or fluoroquinolone-resistant strains of Salmonella enterica serovars Typhi and Paratyphi A has been emerging and becoming major problems throughout the world [21, 23].

Identification of the serovars of Salmonella isolated from blood cultures, the lynchpin of enteric fever surveillance, followed by biochemical testing, is problematic in developing countries. Confirmation of typhoid or paratyphoid fever can also be is made by serological (Widal) test. The diagnostic significance, as determined by Widal test, can be confirmed only when a 4-fold increase in antibodies is demonstrated over a 2 to 3 week period.

Classical methods for confirmation of serotype of Salmonella require high quality O and H typing antisera, reagents that are costly and can be difficult to obtain regularly. These tests also require specialized laboratory set up as well as technical expertise which is possible only in reference laboratories meant for typing purpose. Accordingly, reference and research laboratories in developing countries, as in industrialized countries, are turning to PCR based methods as a consistent, high-throughput approach for typing of etiologic agents [21].

Several PCR methods have been developed to detect Salmonella spp. in clinical, food and environment samples with the aim of improving diagnosis of the infection.

In the present study, PCR standardized for hisJ (hto) gene result in specific amplicon of 495-bp. In positive PCR cases of typhoid (total 15), a single band of 495bp corresponding to hisJ (hto) gene was obtained. The PCR assay was found to be highly specific and the true positive typhoid cases (n = 9) are positive of Widal test, blood culture and/or PCR. PCR was sensitive than blood culture for diagnosis of typhoid fever since the sensitivity and specificity of PCR are 92.86 % and 98.97% respectively and the sensitivity and specificity of blood culture are 87.5% and 96.90% respectively. The positive and negative predictive values of PCR are 93.66% and 97.01% respectively and the positive and negative predictive values of blood culture are 82.03% and 95.31% respectively. The accuracy (%) of PCR and blood culture are 96.52% and 93.04% respectively. The standard error of PCR and blood culture are 0.006 and 0.008 respectively.

We concluded that PCR was sensitive than blood culture for diagnosis of typhoid fever.

The results of molecular characterization are on similar lines observed by Patil et al. (2018) [22].

Most methods have been directed against all Salmonella, but Haque et al. (1999) developed a nested PCR method specifically to detect S. Typhi [24].

Levy et al. (2008) utilized three sequential PCRs to identify the three classical pathogens that cause enteric fever, Salmonella serovars Typhi, -As Salmonella infection is the leading food-borne disease in developing countries [25].

Mothwala et al. (2014) aimed to develop a simple duplex PCR for concurrent diagnosis and differentiation of human adapted Salmonella serovars Typhi and Paratyphi A from other salmonellae, two duplex PCR assays were developed based on clyA, hisJ and invA genes [21].

Further work is needed to test the developed assays on more number of serovars/strains so that their utility can further be accessed in differentiating human and non-human adapted Salmonella serovars.

In the present study, there was a significant correlation between widal titre and blood culture (p = 0.001) in patients with typhoid fever. There was a non- significant association between widal titre and clinical symptoms, including fever, vomiting and diarrhea, abdominal pain, and constipation in patients with typhoid fever.

The Widal test has got limitations such as the difficulty in interpretation, the need to demonstrate a fourfold rise after a week and necessity of knowing the endemicity of the area. A fourfold rise in the Widal assay titer is therefore generally required for a definite serological diagnosis, and a second serum sample is rarely obtained in regions where enteric fever is a major concern. It is

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indicative of typhoid fever in only 40-60% of patients at the time of admission. Test for the presence of Salmonella antibodies in the patients' serum may be of value in the diagnosis of enteric fever. The patients' serum is tested by agglutination method for its antibody titre against O, H and Vi suspensions of enteric fever organisms, like S. typhi, S. paratyphi A and S. paratyphi B. The test results are difficult to interpret in areas where typhoid and other Salmonelliosis are endemic. The test is most reliable if the interpretation is made against background of baseline agglutinin titer in normal individual and in non-typhoidal fevers common in the region. A Vi agglutination reaction has been used to screen for S. enterica serotype typhi carriers. Its reported sensitivity is 70-80%, with a specificity of 80-95% [26].

Positive result in single widal test by no means always indicate the presence of enteric fever because both false positive and false negative results are common and in interpreting them the following points are to be borne in mind: (l) The serum of some normal (uninfected) persons agglutinate Salmonella suspensions at dilutions up to about 1 in 80, so that titres can not be taken as significant unless they are greater than 80. (2) Persons who have received TAB vaccine may show high titres of antibodies to each of the Salmonella and only if a marked rise of titre of one serotype is observed the result can be regarded as diagnostically significant. H-agglutinins tend to persist for many months after vaccination but, O-agglutinins tend to disappear sooner e.g. within six months. (3) H antibody titre is extremely variable arid subject to nonspecific rise (anamnestic reaction). H-antibody is used to differentiate Salmonella species [27]. (4) Nonspecific antigens, such as fimbrial antigen, may be present in test suspensions and then give false positive results by reacting with an agglutinin in the serum of some un-infected individuals. (5) The Widal reaction is positive in many healthy carriers. A negative reaction does not exclude the carrier state, but a positive reaction, particularly a Vi titre of 10 or higher, is said to be helpful for the recognition of the carrier [26].

The sensitivity of blood culture is higher in the first week of the illness which is reduced by prior use of antibiotics, and increases with the volume of blood cultured and the ratio of blood to broth [28]. The rate of isolation of bacteria from blood approaches 90% in untreated patients in the first week; however, the figure falls to less than 50% by the third week. Cultures have also been made from the buffy coat of blood, streptokinase-treated blood clots, intestinal secretions (with the use of a duodenal string capsule), and skin snips of rose spots. Multiple positive blood culture results are 73-97% specific for typhoid fever. Large-volume blood culture and clot culture after serum removal increase sensitivity [26].

Diagnosis of typhoid fever is normally made in all developed countries principally by blood cultures and also by stool/urine culture and serological testing. The sensitivity, specificity, positive and negative predictive values, likelihood ratios were same for PCR based detection of S. typhi in blood and urine samples.

Furthermore in the present study, there was a significant correlation between widal titre and PCR ($p = 0.001$) in patients with typhoid fever.

Nested PCR had higher efficacy in detecting typhoid fever than Widal test, blood and urine cultures. A cut off titer of TO > 1:80 and/or TH > 1:160 was found to have better diagnostic value in this region [29].

In conclusion, we recommend using PCR as an alternative to blood culture for diagnosis of typhoid fever. Further studies are required to compare different techniques. We also recommend performance of a large scale study to test this PCR technique for screening for tyhoid fever in Egypt.

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