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Evaluation of Blood Culture, Widal Reaction, qPCR, and Immunochromatographic Test in the Diagnosis of Typhoid Fever and Its Co-Infection with Malaria among Hospitalized Febrile Patients in Lagos, Nigeria

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ABSTRACT

Background: Typhoid fever is endemic in Nigeria, and there are no high-quality routine diagnostic tests. Immunodiagnostic assays, blood culture, and quantitative real-time polymerase chain reaction (qPCR) were used to study typhoid fever co-infection with malaria. .
Materials & Methods: Blood samples of 125 patients were taken from April to August 2021. The samples were analyzed using standard microbiological methods such as immunodiagnostic assays and qPCR, whereas malaria parasitemia was examined using microscopy.
Findings: In the study, the Widal test (WT) showed that 28.8 and 32.8% of the patients had antibodies against O-antigen and H-antigen at a cut-off titre of 1:160, respectively. The immunochromatographic test (ICT) indicated that 16% had IgM antibodies, and 18.4% had both IgM and IgG antibodies, suggesting a recent typhoid infection. Various bacterial pathogens were identified in patients with positive WT and ICT results, including *Salmonella* species, *Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus* species, and *Proteus mirabilis*. Eight *Salmonella* strains were confirmed through invA gene detection using qPCR, none of which were *S*. Typhi and *S*. Paratyphi A. Both WT and ICT exhibited low sensitivity but high specificity ranging from 73.6 to 81.6% with negative predictive values of 100%. Additionally, the results showed a prevalence rate of 65.5% for malaria parasitemia and 9.8% for non-typhoidal *Salmonella*-associated bacteraemia co-infection with malaria.
Conclusion: This research once again highlighted the limited diagnostic accuracy of both immunochromatographic and Widal tests. The presence of concurrent infections involving malaria and other bacterial pathogens further exacerbates the inadequacies of these diagnostic methods.

Keywords: Typhoid fever, Malaria, Microscopy, qPCR, Widal test, Immunochromatographic test

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Introduction

Typhoid or enteric fever is a severe systemic infection that poses a significant threat to public health in resource-limited areas and in some developing countries where typhoid fever remains endemic [1, 2]. In 2017, about 10 million typhoid fever cases were reported worldwide, resulting in more than 166,000 deaths [3]. Children below five years account for approximately 12.6% of typhoid fever cases, while the incidence rate is higher in children between 5 and 9 years. About 55.9% of cases occur in children younger than 15 years, and 56.3% of cases occur among male subjects, with a global case fatality rate of 0.95% [3, 4]. Typhoidal *Salmonella* consists of *Salmonella* Typhi, which causes typhoid or enteric fever, and *Salmonella* Paratyphi A, B, and C, which cause paratyphoid fever, respectively; these complications are collectively known as 'typhoid' and "enteric fever," which are often used interchangeably [5, 6]. Although typhoid fever has been virtually eradicated in high-income countries, the disease still persists as a significant public health challenge in a substantial number of low- and middle-income countries (LMICs) [7, 8]. The clinical presentations of the disease include (but are not limited to) headache, leukopenia, malaise, transient rashes (rose spots), and febrile illnesses (bacteraemia), which are associated with prolonged fever and may result in complications such as gastrointestinal bleeding, bradycardia, septic shock, and even death if left untreated [2, 9]. Given that the symptoms of typhoid fever are sometimes similar to those of other febrile infections, diagnosing the disease based solely on clinical presentations could be challenging. As a result, rapid and accurate diagnosis of typhoid at the onset of the disease is critical not only for typhoid fever management but also for identifying potential carriers [10]. Isolation of *S*. Typhi from bone marrow, blood, and stool samples of an infected patient remains the definitive diagnosis of typhoid fever, while bone marrow culture remains the gold standard [5]. However, these techniques are expensive, and facilities for such techniques are often unavailable in LMICs [10, 11].

Despite the availability of several diagnostic methods, accurate diagnosis of typhoid fever remains a challenge. The Widal agglutination test, developed by F. Widal in 1896, and the immunochromatographic test (IgG: immunoglobulin G/IgM: immunoglobulin M) are serological methods widely used in low-resource settings to diagnose typhoid fever. These techniques are not only fast, cost-effective, and simple to use but also require little training [12]. The basis of the Widal test is an agglutination reaction between flagella (H) and somatic lipopolysaccharide (O) antigens of *S*. Typhi with antibody responses, but the cross-reactivity of these antigens with some members of the *Enterobacteriaceae* is a major drawback [13]. *S. enterica* subsp. *enterica* serovar Typhi 50 kDa outer membrane protein (OMP) antigen is the target of the immunochromatographic test, an enzyme immunoassay (EIA) that measures IgM and IgG antibody responses [14]. In Nigeria, as in many other LMICs, the diagnosis of typhoid is still largely based on the Widal agglutination test with an acceptable but debatable cut-off titre, while immunochromatographic tests are mostly used in general hospitals in Lagos, Nigeria. Quantitative real-time polymerase chain reaction (qPCR) is a typing method for characterising *S*. Typhi, but it is rarely used as a routine diagnostic method in Nigerian hospitals.

Furthermore, given the comparable clinical presentations of malaria and other acute febrile infections (AFIs), their differentiation and differential diagnosis could be challenging in low- and middle-income countries (LMICs). For children under five

years of age, malaria is a leading cause of morbidity and mortality, considering that its transmission is extremely high in Africa. Unfortunately, most patients presenting with febrile illness are presumptively treated as malaria cases [15].

Objectives: In this study, the performance of blood culture and qPCR was compared with those of Widal agglutination test and immunochromatographic assays to examine malaria co-infection with typhoid fever in febrile patients.

Materials and methods

Study population and case definition: A total of 125 hospitalised patients with a clinical prognosis of typhoid fever were enlisted to receive treatment at various public health facilities in Lagos state, Nigeria from April to August 2021. The patients' demographic information and medical history were gathered.

Inclusion criteria: Patients were considered to have a febrile illness if they had a body temperature higher than 37.9 °C during the previous three days and experienced one or more of the following symptoms: chills for up to five days, diarrhoea, headache, vomiting, nausea, and anorexia

Exclusion criteria: All patients who presented with febrile illness but had other clinical prognoses such as respiratory infection or other illnesses not related to typhoid were excluded. In addition, patients who had a history of antibiotic therapy within 1-2 weeks prior to sampling were excluded.

Ethical approval/Permission: The 1964 Helsinki Declaration and its later amendments and comparable ethical standards were followed to obtain ethical approval from the Human Research and Ethics Committee of Lagos State University Teaching Hospital with reference number LREC/06/10/1012 as well as permission from the Lagos State Health Service Commission with reference number LSHSC/2222/VOL.VC/352 prior to the enrolment of patients. All participants and/or their legal guardians provided written informed consent.

Sample size: The following formula is used to estimate the sample size for descriptive investigations that require a minimum sample: N = Z2 p (1-p)/d2, where d is the degree of precision at 5% (0.05); p is the expected prevalence of the disease, which is equal to 1.6% as reported by Akinyemi and colleagues (2021) [16]; Z is the statistic corresponding (standard error) to a 95% confidence level, which is equal to 1.96; and N is the sample size (73.75872).

Sample collection and processing: About ten mL of the blood of all participants was taken aseptically regardless of their age. Half of each blood sample (5 mL) was dispensed into bottles containing anticoagulant and used for blood culture and malaria testing, and the other half was poured into plain bottles and used for serological testing. The blood samples in the plain bottles were allowed to clot and retract. The clotted blood samples were centrifuged for 10 min at 4000 rpm in a bucket centrifuge (Gallenkamp, Cambridge, UK). Sera were separated and kept frozen at –20 ˚C until used.

Immunodiagnostic assays: The frozen sera were thawed. A Widal kit from Chromatest Febrile Antigens Kits (Linear Chemicals, Spain) was used to perform slide agglutination and semi-quantitative tube titration methods and rapid chromatographic immunoassay (Typhoid IgG/IgM Rapid Test Kit, Vitrosens Biotechnology, Turkey).

Slide agglutination test: About 0.05 mL of each somatic (O, OA, OB, OC) and flagella (H, HA, HB, HC) antigen was dispensed in a different position on a white tile provided. A drop of the patient's serum was added to the antigen and properly mixed. The mixture was rocked for 5 min. A serum-antigen

mixture showing visible agglutination was reported as reactive.

Standard tube titration method: The Widal assay was performed using a 2-fold serial dilution according to the manufacturer's protocol (Chromatest Febrile Antigens Kits, Linear Chemicals, Spain).

Rapid chromatographic immunoassay (Vitrosens Biotechnology, Turkey): A pipette dropper was used to drip the sera of febrile patients, it was held vertically, and one drop of serum was dropped into the specimen area. Then two drops of buffer were added to the same specimen area and allowed to stand; the results were reported after 15 min. The presence of coloured lines in the test and control areas was reported as positive.

Blood film staining (Giemsa) and microscopy: Two films (thick and thin) of each anti-coagulated blood sample were smeared on an oil-free glass slide and air-dried. After fixation in pure methanol, the smear was allowed to air dry.

The thin film was inserted into a jar containing freshly-prepared working Giemsa stain (Giemsa stain stock: 1 mL, working Giemsa buffer: 39 mL, 5% Triton X-100: 2 drops) for 45 min.

The slide was removed from the jar containing the stain and rinsed by dipping it four times in the Giemsa buffer. The slide was dried upright on a rack. The thick film was covered with a 10% Giemsa stain for 10 min. The stain was discarded, and the smear was left in Giemsa buffer for five min. The slide was placed on a rack to dry upright. A drop of immersion oil was poured on the smear and viewed under a microscope using a 100× oil immersion objective lens. A thick film slide was considered negative when no parasite was detected after examining a minimum of 100 high-power fields. A slide containing a smear was considered positive when parasite species as well as their stages were visible [17].

Blood culture: In this step, 3 mL of aseptically-taken blood samples were dispensed into bottles containing 27 mL of brain heart infusion (BHI) broth (LAB M, Lancashire, UK) and mixed by gentle inversion. The blood culture was incubated aerobically at 37 °C for one week as described by Akinyemi et al. (2021) [18]. Bottles showing turbidity were sub-cultured on solid media such as bismuth sulphite agar (BSA), MacConkey agar, *Salmonella-Shigella* agar (SSA) (LAB M, Lancashire, UK), xylose lysine agar (XLD), and blood agar (Himedia, India). The inoculated plates were incubated at 37 °C for 18–24 hours.

Bacterial identification: Following the manufacturer's instructions, the analytical profile index (API) (Institut Mérieux, Marcy l'Etoile, France) was used to identify the isolated bacteria. In order to create a profile index code, the outcomes of multiple biochemical media were analyzed. The codes generated were computed in the API-WEB database (https://apiweb.biomerieux.com), which generates the organism's name.

DNA extraction: DNA extraction was carried out using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described by Akinyemi et al. (2021) [18].

In short, *Salmonella* isolate stock cultures kept at 4 °C was sub-cultured in BHI. The inoculated plates were incubated at 37 °C for 18 to 24 hours.

A loop full of the culture plate of the fresh bacterial isolate (which was 18–24 hours old) was taken out, and 180 µL of buffer ATL was added to it in a 2 mL tube. Thereafter 20 µL of proteinase K was added, mixed, and incubated at 56 °C for two hours.

Following the incubation period, 200 µL of buffer AL was added and stirred for 15 seconds by pulse-vortexing. After another 10 min of incubation at 70 °C, 200 µL of

%96 ethanol was added to the mixture and stirred for 15 seconds by pulse-vortexing. The slurry was slowly poured into the marked QIAamp mini spin columns. The 2-mL collection tube held the column. After closing the cap and centrifugation at 6000 x g for one min, the tube holding the filtrate was thrown away, and the QIAamp mini spin column was taken out and placed in another fresh 2-mL collection tube.

After adding 500 µL of buffer AW1, closing the cap, and centrifuging for one min at 6000 x g, the tube holding the filtrate was thrown away, and the QIAamp mini spin column was taken out and placed in another fresh 2-mL collection tube.

The QIAamp mini spin column was filled with 500 µL of buffer AW2 and centrifuged at full speed (20,000 x g) for 3 min. The filtrate in the collection tube was discarded, and the QIAamp mini spin column was taken out and placed in a sterile 1.5-mL microcentrifuge tube. After opening the QIAamp mini spin column and adding 200 µL of buffer AE, the mixture was left at room temperature for one min and then centrifuged at 6000 x g for one min.

After all these steps, the QIAamp mini spin column was discarded, and the filtrate was saved as a DNA extract. The quantity and quality of the extracted DNA sample were measured using a nanoDrop 1000 spectrophotometer (Beckman Countler, Thermo Fisher Scientific, USA).

Quantitative real-time PCR detection of *inv*A, *S*. Typhi, and *S*. Paratyphi A: *S. Typhi*, *S. Paratyphi* A, and *invA* genes were amplified and detected with previously published primer sets: *invA*-F:5'-ACCACGCTCTTTCGTCTGG-3',*invA*-R:5'-GAACTGACTACGTAGACGCTC-3' [19],STY0313/SPA2475/t2576-F:5-CTTGACGTACCGGTAGAGATATACTGGCT-3, and STY0316/t2574-R:5-CTTGACGTACCGGATATACTGGCT-3[20]. Quantitative real-time PCR experiments were carried out using a Qiagen rotor gene Q 2plex thermocycler (Qiagen, Hilden, Germany) with 36-well reaction tubes as previously described by Fakorede et al. (2023) [21]. Briefly, 12.5 µL of 2×SYBR®Green Taq PCR master mix (Qiagen, Hilden, Germany) was dispensed into 0.1-mL PCR tubes (Eppendorf, Germany). Then1.5 µL of both forward and reverse primers (Inqaba Biotec West Africa, Nigeria) and 10.5 µL of nucleic acid-free water (Qiagen, Hilden, Germany) were added to the mixture. Lastly, 1.5 µL of bacterial DNA (approximately 10^4 copies of gDNA) was calculated according to the genome size of each target *Salmonella* isolate using the following formula and added to the mixture and amplified.

$$CN = \frac{M \times AC}{MW \times G6}$$

PCR amplification was performed under the following thermal cycling conditions: an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s. Internal reference dye (ROX) signal was used to normalize the fluorescent reporter signal. Threshold limit setting was done automatically. A" no template" control (NTC) was run in parallel with each reaction. Positive signals were only those that were produced by SYBR® Green qPCR analysis and showed amplification above the threshold level. Conversely, signals that had no Ct (cycle threshold) value were regarded as negative.

Statistical analysis: A Microsoft Excel 2015 spreadsheet was used to record data, which were then analyzed using Epi Info (Version 6.0, USD; Stone Mountain, GA) and the Statistical Package for Social Sciences (SPSS) for Windows (Version 15.2; Chicago, IL). Cohen's kappa statistical analysis was used to demonstrate the agreement between slide agglutination and tube titration methods. The frequency of *Salmonella* strains as

well as associated factors among feverish and diarrheagenic patients' in particular healthcare settings were determined using Chi-square. If the p-value was less than 0.05, the statistical test was deemed significant.

Sensitivity, specificity, and positive and negative predictive values: The sensitivity, specificity, and positive and negative predictive values (PPV and NPV) were calculated using the MedCalc Software Ltd. diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php) (Version 22.030). Sensitivity, specificity, PPV, and NPV of Widal agglutination test and Immunochromatographic test were calculated using the following formulas:

Sensitivity: $\frac{TP}{TP+FN}$

Specificity: $\frac{TN}{TN+FP}$

PPV: $\frac{\text{sensitivity x prevalence}}{\text{Sensitivity x prevalence} + (1\text{-specificity}) + (1\text{-prevalence})}$

NPV: $\frac{\text{specificity x (1- prevalence)}}{(1\text{-sensitivity}) \text{x prevalence} + \text{specificity x} (1 - \text{prevalence})}$

Where TP is truth positive results, TN is truth negative results, FP is false positive results, FN is false negative results, PPV is positive predictive value, and NPV is negative predictive value.

Findings

Sero-diagnosis of typhoid fever: A total of 58.4% (n=73) of participants' sera were reactive in the Widal slide agglutination test, of which 26.4% (n=33) reacted to both O and H antigens. Moreover, 36.8% (46 of 125) of the blood samples were positive in the immunochromatographic test (ICT), of which 16% (20 of 125) were positive for IgM, 2.4% (3 of 125) for IgG, and 18.4% (23 of 125) for both IgM and IgG (Table 1).

Anti-S. Typhi antibody titres in sera of febrile patients: The standard tube titration method revealed anti-TO antibody reaction in 45 (36%) febrile patients' sera with positive slide reaction, while anti-TH antibody reaction was observed in 61 (48.8%) patients' sera with positive slide reaction. Using an antibody cut-off titre of 1:160, 80% (36 of 45) had anti-TO antibody reactions, and 67.2% (41 of 61) had anti-TH antibody reactions, indicating recent infection (Table 2).

Cohen's kappa statistical analysis used to demonstrate the agreement between slide agglutination and tube titration methods revealed a moderate agreement of 72.28% with a Cohen's k of 0.4 (Table 2).

Typhoid fever and malaria co-infection among febrile patients: Of the 125 participants screened for malaria, 65.6% (n=82) were positive for *Plasmodium falciparum* by microscopy.

Table 1) Comparative results of Widal agglutination (slide) and immunochromatographic tests in serodiagnosis of typhoid fever

Assay	Immunodiagnostic Assay					
Detection Methods	Widal Agglutination Test (Slide) (N=125)			Immuno-Chromatographic Assay (N=125)		
Antigen	O A	H A	O & H A	IgM	IgG	IgM/IgG
Number positive	12 (9.6%)	28 (22.4%)	33 (26.4%)	20 (16%)	3 (2.4%)	23 (18.4%)
Number negative	113 (90.4%)	97 (77.6%)	92 (73.6%)	105 (84%)	122 (97.6%)	102 (81.6%)

Table 2) Frequency distribution of anti-*S.* Typhi antibody titres in sera of febrile patients with reactive slide agglutination

O-Antigen				H-Antigen				Statistical Analysis
Tube Titre	Frequency	Reactive Slide (N=45)	Total Sera Screened (N=125)	Tube Titre	Frequency	Reactive Slide (N=61)	Total Sera Screened (N=125)	Cohen's k Test
1:20	0	0	0	1:20	2	3.2%	1.6%	Percentage of agreement: 72.28%. Cohen's k: 0.4.
1:40	0	0	0%	1:40	4	6.6%	3.2%	
1:80	9	20%	7.2%	1:80	14	22.95%	11.2%	
1:160	17	37.8%	13.6%	1:160	20	32.8%	16%	
1:320	11	24.4%	8.8%	1:320	16	26.2%	12.8%	
1:640	8	17.8%	6.4%	1:640	5	8.2%	4%	

P. falciparum was detected in 76.7% (56 of 73) of patients with positive Widal reactions and 76.1% (35 of 46) of patients with reactive ICTs (Table 3). *P. falciparum* was detected in different titre values, it was mostly detected in patients with titre values of 1:80 (23 of 82, 28.05%). The prevalence of *P. falciparum* was (71.4%, 30 of 42) higher among patients in the age group of 1-15 years than in other age groups.

Meanwhile, a prevalence of 90.7% (49 of 52) was recorded for *P. falciparum* among patients with pyrexia of unknown origin, which was found to be higher compared to other clinical data. A high prevalence of 89.1% for *P. falciparum* was also detected among patients with positive IgM (Table 3).

Bacterial pathogens in seropositive patients and malaria co-infection: In this study, 23 out of 125 (18.4%) febrile patients screened for typhoid fever had positive blood cultures. Of the 23 bacterial pathogens detected, 6.4% (n=8) were identified as *Salmonella* spp., 0.8% (n=1) as *Streptococcus* spp., 1.6% (n=2) as *Staphylococcus aureus*, 4.8% (n=6) as *Proteus mirabilis*, and 4.8% (n=6) as *Klebsiella pneumoniae* (Figure 1). *P. falciparum* co-infection was observed in six out of eight *Salmonella* positive cases and three out of six *Klebsiella* positive cases (Figure 1).

All *Salmonella* isolates were found to harbour the *inv*A gene using qPCR with Ct values ranging from 20.40 to 27.60, although all eight *Salmonella* species detected were non-typhoidal (Table 4).

Bacterial pathogens were detected in various titre values ranging from 1:40 to 1:640 with Widal test and positive ICT, including *Salmonella* spp., *P. mirabilis*, *S. aureus*, and *K. pneumoniae* (Table 5).

Sero-prevalence of typhoid fever: The seroprevalence of typhoid fever among male and female participants was 24.1 and 28.2% in the Widal test and 33.3 and 35.2% in the ICT. The seroprevalence of typhoid fever was higher among patients in the age group of 0–15 years (33.3%) in both Widal agglutination test and ICT (Table 6).

Sensitivity and specificity of immunodiagnostic assays: The screening methods showed lack of sensitivity (0%) with no positive predictive value (PPV) for both WT and

Table 3) Prevalence of *Plasmodium falciparum* co-infection in typhoid fever patients

Variables	Categories	No Tested	Positive Malaria	*P. falciparum* Detected in Different Titres Value (O and H- Antigens)						*P. falciparum* Detected in Positive Immunochromatographic Test			
		n-125	n-82	Neg. Widal	1:20	1:40	1:80	1:160	1:320	1:640	Neg. ICT	Pos. IgM	Pos. IgG	Pos. IgM & IgG
Gender	Male	54	30 (55.6%)	6	1	1	7	6	7	2	17	8	1	4
	Female	71	52 (73.2%)	17	0	3	16	9	4	3	31	9	3	10
Age	0-15	42	30 (71.4%)	7	0	1	12	4	3	3	12	9	1	2
	16-30	33	23 (69.7%)	6	0	2	5	6	3	1	11	5	0	7
	31-45	22	14 (63.6%)	7	1	1	2	1	2	0	5	2	1	3
	46-60	16	9 (56.3%)	3	0	0	2	2	1	1	5	1	1	2
	≥ 60	12	6 (50.0%)	0	0	0	1	2	3	0	4	2	0	0
Prognosis	PUO	54	49 (90.7%)	12	1	4	10	11	7	4	22	12	2	2
	Anaemia	12	8 (66.7%)	0	0	0	5	1	2	0	5	3	1	3
	Headache	16	10 (62.5%)	4	0	0	6	0	0	0	9	6	3	5
	Abdominal pain	12	4 (33.3%)	1	0	0	0	3	0	0	2	0	2	0
	Diarrhoea	16	8 (50%)	6	0	0	0	0	1	1	1	1	0	0
	Vomiting	10	3 (30%)	0	0	0	2	0	1	0	1	1	1	0
	Coated tongue	1	0 (0%)	0	0	0	0	0	0	0	0	0	0	0
	Constipation	4	0 (0%)	0	0	0	0	0	0	0	0	0	0	0

Dark blue Coloured boxes indicate the highest occurrence of *Plasmodium falciparum* in specific titre values and antibodies among the variable

ICT methods. The study, however, revealed a high specificity of 73.60 and 81.60% and a negative predictive value of 100% for WT and ICT methods, respectively (Table 7).

In WT and ICT, both true positive (TP) and false negative (FN) values were zero, but

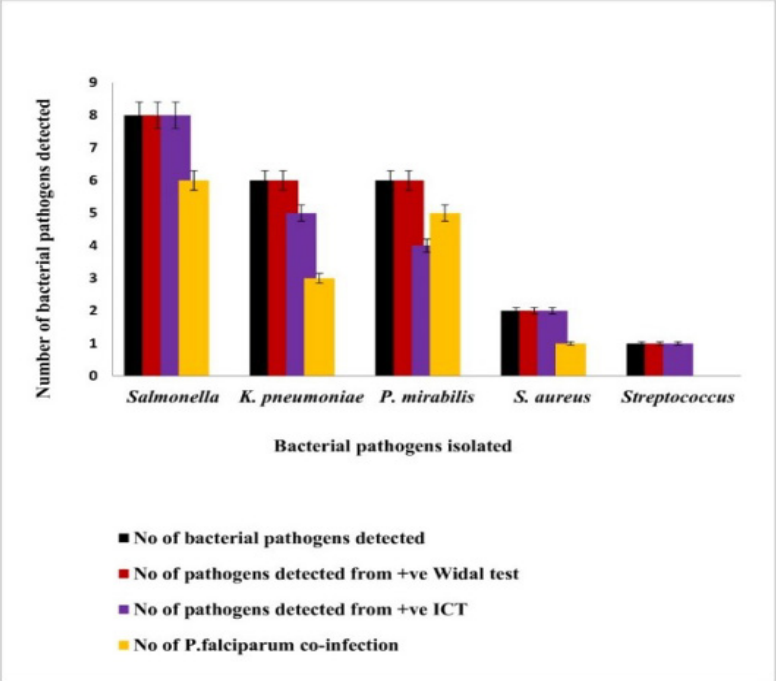

Figure 1) Bacterial pathogens isolated from blood cultures of Widal and ICT seropositive patients with suspected typhoid fever and malaria co-infection

Table 4) Culture and qPCR detection of *Salmonella* spp occurrence in blood sample of patients positive for immunodiagnostic assay

Isolate ID	Meta Data			Culture	qPCR					
	Age	Sex	Clinical Prognosis		*invA*	*Ct value*	*S. Typhi*	*Ct value*	*S. Paratyphi*	*Ct value*
SL001	4	F	Pyrexia of unknown origin	*Salmonella spp*	+	20.40	-	-	-	-
SL002	5	M	Pyrexia of unknown origin	*Salmonella spp*	+	23.39	-	-	-	-
SL003	8	M	Pyrexia of unknown origin	*Salmonella spp*	+	21.04	-	-	-	-
SL004	9	F	Pyrexia of unknown origin	*Salmonella spp*	+	26.19	-	-	-	-
SL005	16	M	Pyrexia of unknown origin	*Salmonella spp*	+	27.60	-	-	-	-
SL006	22	M	Abdominal pain	*Salmonella spp*	+	21.08	-	-	-	-
SL007	33	F	Coated tongue	*Salmonella spp*	+	22.16	-	-	-	-
SL008	49	M	Pyrexia of unknown origin	*Salmonella spp*	+	23.41	-	-	-	-
Pos. control	NA	NA	NA	*S. Typhi* ATCC 6539	+	18.07	+	16.04	-	-
Pos. control	NA	NA	NA	*S. Paratyphi* ATCC 9150	+	18.07	-	-	+	24.78
No template control	NA	NA	NA	NA	-	-	-	-	-	-

NA: Not applicable

Table 5) Correlation between positive immunodiagnostic assay and positive blood culture in serodiagnosis of typhoid fever

Organisms Detected	Number of Isolates N=23		Antibodies and Widal titre value of patients with positive blood cultures						
			Antibodies	1:20	1:40	1:80	1:160	1:320	1:640
Salmonella spp	8(34.8%)	Widal	O	0	0	0	1	2	1
			H	0	0	0	0	0	1
			O&H	0	1	0	1	0	2
		ICT	IgG	0	0	1	0	0	0
			IgM	0	0	0	1	4	0
			IgM+IgG	0	0	0	1	1	0
Proteus mirabilis	6(26.1%)		O	0	0	0	0	1	1
			H	0	0	2	0	2	0
			O&H	0	0	1	0	2	0
			IgG	0	0	0	1	0	0
			IgM	0	0	0	1	0	0
			IgM+IgG	0	0	0	1	1	0
Staphylococcus aureus	2(8.7%)		O	0	0	0	0	0	1
			H	0	0	0	0	0	0
			O&H	0	1	0	1	0	0
			IgG	0	0	1	0	0	0
			IgM	0	0	0	0	0	0
			IgM+IgG	1	0	0	0	0	0
Streptococcus spp	1(4.3%)		O	1	0	0	0	0	0
			H	0	0	0	0	0	0
			O&H	0	0	0	0	0	0
			IgG	0	0	0	0	0	0
			IgM	0	1	0	0	0	0
			IgM+IgG	0	0	0	0	0	0
Klebsiella pneumoniae	6(26.1%)		O	1	0	0	0	0	1
			H	0	0	1	0	0	0
			O&H	0	0	1	0	2	0
			IgG	0	0	0	1	1	0
			IgM	0	0	1	0	0	0
			IgM+IgG	0	0	0	1	0	1

true negative (TN) values were equal to 92 and 102, and false positive (FP) values were equal to 33 and 23, respectively, and *S*. Typhi and *S*. Paratyphi A strains were not detected at all.

Discussion

Typhoid fever, which lacks pathognomonic clinical markers, is a systemic disease with a variety of multisystem clinical presentations [22]. Typhoid fever is a serious public health threat, especially in resource-limited areas. A number of rapid diagnostic methods with easy access and quick turnaround time have been developed for typhoid detection

Table 6) Seroprevalence of typhoid fever among febrile patients and their associated predictors

	Categories	No Tested	Widal +	Seroprevalence	Salmonella Detected	POSISWAT	No Tested	ICT +	Seroprevalence	Salmonella Detected	POSISICT
Gender	Male	54	13	24.10%	5	38.50%	54	18	33.30%	5	27.80%
	Female	71	20	28.20%	3	15%	71	25	35.21%	3	12%
Age distribution	0-15 yrs.	42	14	33.30%	4	28.60%	42	24	33.33%	4	16.70%
	16-30 yrs.	33	9	27.30%	2	22.20%	33	10	30.30%	2	20%
	31-45 yrs.	22	5	22.73%	1	20.00%	22	6	27.27%	1	16.70%
	48-60 yrs.	16	3	18.60%	1	33.30%	16	2	12.50%	1	50%
	≥ 61 yrs.	12	2	16.70%	0	0.00%	12	1	8.33%	0	0%
Clinical prognosis	PUO	54	22	40.70%	6	27.30%	54	14	26%	6	43%
	anaemia	12	6	50%	0	0.00%	12	5	41.70%	0	0%
	Headache	16	12	75%	0	0.00%	16	7	43.80%	0	0%
	Abdominal pain	12	6	50.00%	1	16.70%	12	4	33.33%	1	25%
	Diarrhoea	16	4	25.00%	0	0.00%	16	3	18.80%	0	0%
	Vomiting	10	6	60.00%	0	0.00%	10	8	80%	0	0%
	Coated tongue	1	1	100.00%	1	100.00%	1	1	100%	1	100%
	Constipation	4	1	25.00%	0	0.00%	4	1	25%	0	0%

POSISWAT: Prevalence of Salmonella in seropositive Widal test
POSISICT: Prevalence of Salmonella in seropositive ICT
ICT: Immunochromatographic test

Table 7) Sensitivity and specificity of immunodiagnostic assay in seroprevalence of typhoid fever

Statistic	Value O & H	95% CI	Value IgM & IgG	95% CI
Sensitivity	0%		0%	
Specificity	73.6%	64.97% to 81.08%	81.6%	73.68% to 87.69%
Disease prevalence	0%		0%	
Positive Predictive Value (PPV)	0%		0%	
Negative Predictive Value (NPV)	100%	96.07% to 100%	100%	96.45% to 100%

in recent years. This research investigated the seroprevalence of enteric fever and its co-infection with malaria parasitemia among febrile patients. This study revealed an overall seroprevalence of 58.4% (73 of 125) for typhoid fever using the Widal slide agglutination test. Although 26.4% of febrile participants' sera reacted to both somatic (O) and flagella (H) antigens, only 9.6 and 22.4% reacted to O-antigen and H-antigen alone, respectively. This study showed an increase in anti-H antibodies compared to the antibody response against O antigen, which could be due to the slow increase of anti-H antibody titre in acute typhoid fever. The use of the standard tube titration method with a cut-off value of 1:160 for antibody titres against both flagella and somatic antigens to analyse serum samples with reactive slide agglutination test showed that 36 (28%) and 41 (32.8%) patients had recent O and H antigen-induced typhoid infection because of the endemic nature of the disease in Nigeria. Cohen's kappa statistical analysis showed a moderate agreement of 72.28% with a Cohen's k of 0.4% between slide agglutination and tube titration methods. However, the Chi-square test results showed an insignificant relationship between Widal slide agglutination test and tube titration methods (Chi-square: 0.0501 and p: .999997 for somatic (O) antigen; Chi-square: 0.0137 and p: .999977 for flagella (H) antigen). This study showed a lower seroprevalence of typhoid fever among male participants (24.1%) compared to female participants (28.2%) using the Widal agglutination test. The seroprevalence of typhoid fever (33.3%) was higher among participants in the age group of 1-15 years compared to other age groups, with an average age of 5.6 years, a standard deviation (SD) of ±3.58, a standard error of the mean(SEM) of 0.96, and a margin of error of ±5.57+1.574 at a 95% confidence level.

This study demonstrated the lack of sensitivity of the screening methods with a 0% positive predictive value (PPV). This study results are in contrast with those of other studies, indicating 68.3% seroprevalence and 100% sensitivity in Ethiopia [23] as well as 66.7% sensitivity, 81.6% specificity, and 91.8% negative predictive value for Widal agglutination test in India [11]. In this study, a lower specificity of 73.6% and a higher negative predictive value of 100% were obtained for the Widal test. In this study, the positive predictive values obtained for both O and H antigens were abysmally low at 0% compared to the values obtained for O (2.7%) and H (3.02%) antigens in another study by Wasihun et al. (2015) [23]. The low positive

predictive value recorded in this study could be attributed to the negative culture of *S*. Typhi and *S.* Paratyphi A., which affects the positive predictive value. Therefore, in the absence of effective methods such as culture and molecular methods, the Widal slide agglutination test should be used with caution to diagnose typhoid illness, especially in endemic areas, and treatment should not be based solely on the findings of a single sample from the acute period. The seroprevalence of enteric fever using the rapid immunochromatographic test (ICT) was 36.8% (46 of 125) versus 58.4% (73 of 125) recorded using WT. The results also showed the lack of sensitivity of the ICT method with a 0% positive predictive value (PPV), although an 81.60% specificity (from 73.68 to 87.69% at 95% CI) and a 100% negative predictive value (NPV) were obtained for this method. The low PPV and high NPV in this study is a demonstration of the potential for false negative results. IgM and IgG antibodies were distinguished with the ICT kit. The results of this study showed that 16% (20 of 125) of the samples were positive for only IgM, 2.4% (3 of 125) for only IgG, and 18.4% (23 of 125) for both IgM and IgG. When seroconversion occurs, IgG without IgM typically implies past infection, re-emergence of infection, or late-stage disease. Only the presence of IgM (early phase) or both IgM and IgG (middle phase) indicates a current infection. This study recorded a higher prevalence of IgM compared to IgG using ICT, which is consistent with the findings of another study that used a different ICT kit and found more IgM-positive cases [24]. According to previous studies, rapid diagnostic tests (RDT) yield more reliable results than the Widal test in terms of sensitivity and specificity [9, 25]. In a study by Sanjeev et al.(2013)[25], Typhi-dot outperformed the Widal test in terms of sensitivity and specificity, with scores of 100 and 76% as well as 78.78 and 58.82%, respectively. They proposed that in addition to blood cultures, quick tests like Typhi-dot were more beneficial than Widal tests in routine diagnostic services, which is contrary to this study findings demonstrating suboptimal performance of the immunodiagnostic assay in typhoid fever diagnosis. The use of various formats of fast diagnostic test kits from different manufacturers may be the cause of discrepancies in sensitivity and specificity in various studies. The length of time between clinical prognosis and test execution may also affect the uniformity of ICTs. Out of the 125 samples screened, *P. falciparum* was detected in 82 (65.6%) patient samples by microscopy. Among 82 patients with malaria parasitemia, *P. falciparum* was detected in 71.95% (59 of 82) of patient samples with reactive Widal agglutination tests and 28.05% (23 of 82) of patient samples with negative Widal reactions. Similarly, 42.7% (35 of 82) of patients with positive ICT had *P. falciparum* co-infection, while the remaining 57.3% (47 of 82) were ICT-negative patients that were positive for *P. falciparum*. Among patients with positive Widal reaction, *P. falciparum* was detected in different titre values; it was mostly detected in patients with titre values of 1:80 (28.05%). Other titre values in which *P. falciparum* was detected included1:20 (1.25%), 1:40 (4.9%), 1:160 (18.3%), 1:320 (13.4%), and 1:640 (6.1%), respectively. The prevalence of *P. falciparum* was higher (71.4%, 30 of 42) among patients in the age group of 1–15 years compared to other age groups.

Meanwhile, a prevalence of 90.7% (49 of 52) was recorded for *P. falciparum* among patients with pyrexia of unknown origin (PUO), which was found to be higher compared to other clinical prognoses associated with febrile illnesses. *P. falciparum* was also detected in patients with positive IgM (19.5%, 16 of 82),

IgG (4.9%, 4 of 82), and both IgM and IgG (17.1%, 14 of 82), respectively. This study shows that malaria load is strongly correlated with *Salmonella* antibody titres. This study also shows that false-positive results of immunodiagnostic assays are common in patients with malaria and other bacterial infections. *P. falciparum* may share similar immunogenic properties with typhoidal *Salmonella*; thus, *Plasmodium* infections could elicit immunoglobulin generation against *S*. Typhi antigens, resulting in cross-reactivity and false-positive results [26]. The continuous burden of *Salmonella* infection and malaria has been attributed to regional overlaps of these diseases, leading to co-infection of these two pathogens. The prevalence of malaria co-infection with non-typhoidal *Salmonella* (NTS) in individuals with reactive Widal agglutination test in this study was 10.2% (6 of 59). This result is lower than the prevalence of 27% reported in another study by Wilairatana et al. (2021) [27]. Malaria co-infection with other non-*Salmonella* pathogens was also observed among febrile patients with reactive Widal test. The prevalence of malaria and *K. pneumoniae* co-infection was 5.4%; this is comparable to the result (5.6%) reported by Wilairatana et al. (2021)[27], while malaria co-infection with *P. mirabilis* was 8.9% (5 of 56) in this study, which is higher than the result (2.6%) documented in a study by Wilairatana et al. (2021) [27]. Similarly, a prevalence of 1.8% (1 of 56) was recorded for malaria and *S. aureus* co-infection, which is much lower than the prevalence of 44.9% recorded in another study [27]. Variation in the prevalence of co-infection could be linked to regional location, seasonal variation, endemic region, and sample size. This study also revealed a prevalence of 7.3 % (6 of 82) for NTS-associated bacteraemia among patients with malaria parasitemia. This is not uncommon in tropical and subtropical regions where malaria is endemic. NTS and malaria co-infection have been documented in other studies [28, 29]. Co-infection has been documented to predispose these individuals to bacteraemia due to immunosuppression; changes in iron storage metabolism as a result of malaria-induced haemolysis, which may interfere with normal neutrophil function; increased susceptibility; and increased free iron, which may also promote the survival of *Salmonella* spp. [27, 30, 31].

In this investigation, enteric fever had a 58.4% serological prevalence, although only 6.4% (8 of 125) of febrile patients had *S. enterica* subspp. *enterica* infection detected by culture and analytical profile index (API). Quantitative real-time PCR (qPCR) was used as a confirmatory test to investigate the presence of *inv*A genes among *Salmonella* isolates obtained by culture method. The results showed that 100% (8 of 8) of *Salmonella* isolates harboured the *inv*A gene, an indication of the suitability of this gene as a molecular target with potential diagnostic applications for specific detection of *Salmonella*. The *Salmonella inv*A gene is found on *Salmonella* pathogenicity island 1 (SPI-1) and is linked to host epithelial cell invasion. The presence of this gene is an indication of possible invasion of the host's intestinal epithelial cells, which may elicit the host's immunological responses to produce antibodies, these antibodies cross-react with immunodiagnostic assay antigens by binding and agglutination of lipopolysaccharides (LPS) and flagella in the sera of individuals who may have typhoid illness. Quantitative real-time PCR results with the primer sets used revealed that none of the *Salmonella* species detected were either *S*. Typhi or *S*. Paratyphi A, indicating that all *Salmonella* isolates with positive immunodiagnostic assays were non-Typhoidal *Salmonellae*. As demonstrated in this study, *S. enterica* (non-typhoidal) was found in varied titre values:

8.7% in 1:160titres, 8.7% in 1:320 titres, and 17.4% in 1:640 titres. Other bacterial pathogens detected in this study, such as *S. aureus, P. mirabilis, Streptococcus* spp., and *K. pneumoniae*, exhibited increased agglutinin levels against *Salmonella* somatic and flagella antibodies.

Elevated agglutinin levels in patients with culture-negative *Salmonella* could be attributed to a number of factors, including (but not limited to) cross-reactivity as a result of similar antigenic determinants, cross-reactive epitopes, or long-term infection. This study showed that a high Widal titre was present in 65.2% of patients with a negative *S. enterica* culture. For example, *P. mirabilis* was detected in titres of 1:40 (4.3%), 1:160 (17.4%), and 1:640 (4.3%); *Streptococcus* spp. in titre of 1:20 (4.3%); *S. aureus* in titres of 1:160 (4.3%) and 1:320 (4.3%); and *K. pneumoniae* in titres of 1:20 (4.35%), 1:320 (8.7%), and 1:640 (13%). Antibodies that react with various bacterial and non-bacterial infections may account for the high percentage of false-positive results of Widal agglutination and immunochromatographic tests.

The presence of other bacterial pathogens with positive IgM, IgG, or both indicates poor sensitivity of the screening method for diagnosing enteric fever. However, residents of enteric fever-endemic regions, such as Nigeria, are expected to have residual antibodies from previous or recurrent infections caused by enteric fever pathogens or other *Salmonella* serotypes, which may react with the Widal test. As a result of over-diagnosis and over-reporting, the Widal test alone may not be of substantial diagnostic accuracy in endemic regions like Nigeria.

As part of the limitations of this study, we were unable to directly extract DNA from the blood samples of all participants for direct molecular typing and typhoidal *salmonellae* screening.

Conclusion

The polyvalent nature of the relevant antigens used in the serodiagnosis of typhoid fever makes it a straightforward methodology, but it frequently produces inaccurate results. A high prevalence of malaria parasitemia and bacterial co-infection was observed in reactive immunodiagnostic assays, indicating cross-reactivity with immunodiagnostic assay antigens. It is suggested to use more reliable diagnostic methods in these areas, such as stool culture, blood culture, and bone marrow culture that remains as the gold standard, while the use of Widal agglutination and ICT methods for typhoid fever diagnosis should be interpreted with caution.

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