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Widal antibody titre test versus blood culture; which is a better diagnostic for typhoid fever?

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Abstract:

Background: The importance of accurate diagnosis of infectious diseases is central and crucial to the effectiveness of treatment and prevention of the associated long-term complications of such infections. The objective of this study was therefore to determine the accuracy of the Widal antibody titre test in the diagnosis of typhoid fever relative to the gold standard blood culture technique.
Methodology: A total of 40 students attending the Olabisi Onabanjo University Health Services, Ago-Iwoye, Ogun State, Nigeria on account of suspected typhoid fever by positive Widal test (≥ 1/80) and not on antibiotic therapy, were recruited for the study. Stool and blood samples were collected from each participant and analysed at the medical laboratory of the health center using conventional culture techniques and confirmation of isolates by simplex and multiplex polymerase chain reaction (PCR) amplification assays of *hilA* (*Salmonella enterica*), *ipaH* (*Shigella* spp), *rfc* (*Shigella flexneri*) and *wbgZ* (*Shigella sonnei*) genes. Antibiotic susceptibility testing (AST) of isolated bacteria to 10 panel of antibiotics was done using the Kirby Bauer disk diffusion test and interpreted according to the Clinical and Laboratory Standards Institute (CSLI) guideline.
Results: Of the 40 patients with suspected typhoid fever by the Widal test, 9 yielded *Salmonella enterica* giving a 22.5% isolation rate, with *Salmonella enterica* serovar Typhi (*Salmonella* Typhi) confirmed as sole bacterium from blood cultures in 5 (12.5%) patients and co-infection of *Salmonella* and *Shigella* from stool samples in 4 (10.0%) patients. A total of 52 enteric bacteria isolates were recovered from blood and stool samples of the 40 patients made of *Salmonella enterica* 9 (17.3%), *Shigella* spp 20 (38.5%), *S. flexneri* 9 (17.3%) and *S. sonnei* 14 (26.9%). All the enteric isolates were multi-drug resistant (MDR), with resistance rates to the antibiotic panel ranging from 33.3%-100%, and all the isolates were resistant to ceftriaxone and pefloxacin. *Salmonella* isolates were also 100% resistant to nitrofurantoin, ofloxacin and ciprofloxacin; *S. flexneri* were 100% resistant to nitrofurantoin, amoxicillin, cotrimoxazole, ofloxacin and ciprofloxacin; and *S. sonnei* were 100% resistant to nitrofurantoin and cotrimoxazole.
Conclusion: These results showed that only 12.5% of typhoid fever diagnosis by Widal test had *Salmonella* Typhi isolated from their blood cultures while *Salmonella enterica* and *Shigella* spp were isolated from stool samples of other cases. There is need to adopt culture techniques for laboratory diagnosis of febrile illnesses in order to improve treatment regimen. The fact that AST can also be performed with culture technique could further guide antibiotic prescription and reduce the risk of emergence of resistant bacteria.

Keywords: Blood culture; Typhoid fever; Widal test; *Salmonella enterica*; *Shigella* spp.

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Test de titre d'anticorps Widal versus hémoculture; quel est le meilleur diagnostic pour la fièvre typhoïde?

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Résumé:

Contexte: L'importance d'un diagnostic précis des maladies infectieuses est centrale et cruciale pour l'efficacité du traitement et la prévention des complications à long terme associées à ces infections. L'objectif de cette étude était donc de déterminer la précision du test de titre d'anticorps de Widal dans le diagnostic de la fièvre typhoïde par rapport à la technique d'hémoculture de référence.
Méthodologie : Un total de 40 étudiants fréquentant les services de santé de l'Université Olabisi Onabanjo, Ago-Iwoye, État d'Ogun, Nigéria en raison d'une fièvre typhoïde suspectée par un test Widal positif (≥ 1/80) et non sous antibiothérapie, ont été recrutés pour l'étude. Des échantillons de selles et de sang ont été prélevés sur chaque participant et analysés au laboratoire médical du centre de santé à l'aide de techniques de culture conventionnelles et de confirmation des isolats par des tests d'amplification par réaction en chaîne par polymérase (PCR) simplex et multiplex de *hilA* (*Salmonella enterica*), *ipaH* (*Shigella* spp), les gènes *rfc* (*Shigella flexneri*) et *wbgZ* (*Shigella sonnei*). Les tests de sensibilité aux antibiotiques (AST) des bactéries isolées à 10 groupes d'antibiotiques ont été effectués à l'aide du test de diffusion sur disque de Kirby Bauer et interprétés conformément aux directives du Clinical and Laboratory Standards Institute (CSLI).
Résultats: Sur les 40 patients suspects de fièvre typhoïde par le test de Widal, 9 ont révélé Salmonella enterica donnant un taux d'isolement de 22,5%, avec *Salmonella enterica* sérovar Typhi (*Salmonella* Typhi) confirmée comme bactérie unique à partir d'hémocultures chez 5 (12,5%) patients et co-infection de *Salmonella* et *Shigella* à partir d'échantillons de selles chez 4 (10,0%) patients. Un total de 52 isolats de bactéries entériques ont été récupérés à partir d'échantillons de sang et de selles des 40 patients constitués de *Salmonella enterica* 9 (17,3%), *Shigella* spp 20 (38,5%), *S. flexneri* 9 (17,3%) et *S. sonnei* 14 (26,9%). Tous les isolats entériques étaient multirésistants (MDR), avec des taux de résistance au panel d'antibiotiques allant de 33,3% à 100%, et tous les isolats étaient résistants à la ceftriaxone et à la péfloxacine. Les isolats de *Salmonella* étaient également résistants à 100% à la nitrofurantoïne, à l'ofloxacine et à la ciprofloxacine; *S. flexneri* était résistant à 100% à la nitrofurantoïne, à l'amoxicilline, au cotrimoxazole, à l'ofloxacine et à la ciprofloxacine; et *S. sonnei* étaient 100% résistants à la nitrofurantoïne et au cotrimoxazole.
Conclusion: Ces résultats ont montré que seuls 12,5% des diagnostics de fièvre typhoïde par le test de Widal avaient *Salmonella* Typhi isolée à partir de leurs hémocultures, tandis que *Salmonella enterica* et *Shigella* spp ont été isolées à partir d'échantillons de selles d'autres cas. Il est nécessaire d'adopter des techniques de culture pour le diagnostic en laboratoire des maladies fébriles afin d'améliorer le schéma thérapeutique. Le fait que l'AST puisse également être réalisée avec une technique de culture pourrait guider davantage la prescription d'antibiotiques et réduire le risque d'émergence de bactéries résistantes.

Mots clés: Hémoculture; La fièvre typhoïde; test Widal; *Salmonella enterica*; *Shigella* spp

Introduction:

The need to properly identify the aetiological agents of infection is a serious concern considering the elevated trends of false positive test especially in the diagnosis of typhoid fever in Africa (1). This is necessary for appropriate treatment regimen (2), as the quality of microbiology laboratory diagnosis is central and crucial to proper diagnosis of infectious diseases (3). Failure to appropriately provide true positive tests in disease diagnosis represents low sensitivity and specificity of diagnostic tests (4, 5).

In Nigeria where majority of the populace live below the estimated poverty level and are mostly of low socio-economic class that is known to be frequently associated with poor sanitation, and toilet systems among others (6), it is expected that the level of enteric infection transmission could be high (7), and this could easily be misdiagnosed due to the similarities in the manifestations of these infections (4). Infections may also occur concurrently or superimpose on the other (3,8).

Generally, enteric infections continue to be global health challenge, with an estimated 21.6 million people suffering from febrile illnesses and resulting in estimated 200,000 deaths every year (9). In a study conducted by Folorunso et al., (10), isolation of *Salmonella* Typhi was reported in 17.77% of aetiological agents of pyrexia of undetermined origin. It is thus imperative to evaluate the sensitivity and specificity of Widal antibody titre test relative to blood culture by evaluating patients with

significant typhoid antibody titre and comparing with blood culture technique, in order to elucidate the effect of cross-reactions from infections caused by other enteric pathogens such as *Salmonella* Paratyphi and *Shigella*. This study was therefore aimed at evaluating diagnostic accuracy of the Widal test for diagnosis of typhoid fever relative to the blood culture technique.

Materials and method:

Study setting, participants and ethical approval

A total of 40 students attending Olabisi Onabanjo University Health Services, Ago-Iwoye who have been diagnosed with typhoid fever by the Widal test significant antibody titre (≥1/80), were recruited into this study after obtaining their informed consents. Students already on antibiotic treatment were excluded from the study. The study was approved by the Ethics Committee of the Directorate of Health Services, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria.

Sample collection and bacterial isolation

Approximately 2ml of venous blood and 2g of faecal samples were aseptically collected from each consenting participant at the medical laboratory of the Olabisi Onabanjo University Health Services. About 1ml of the blood and a loopful of the faecal samples were aseptically introduced into separate MacCartney bottles containing 10ml each of pre-sterilized Rappaport Vasiliadis and nutrient broth. The mixtures were gently shaken to enhance homogeneity and then incubated at 37^{o}C for an initial period of 48 hours which served as inoculum for subsequent analyses. The inoculum (blood and faecal samples introduced into the Rappaport Vassiliadis broth) was then inoculated onto *Salmonella-Shigella* agar (SSA) and MacConkey agar plates by streaking method after which the plates were incubated aerobically at 37^{o}C for 24 hours. The colonies from the culture plates were sub-cultured on nutrient agar for purity plating prior to identification with molecular technique.

Molecular identification of isolates

The molecular identification of bacterial isolates from suspected typhoid fever patients was carried out in a polyphasic manner. First, each of the isolated bacterium was first pre-incubated at 99°C for 20 mins and DNA extracted using QIAamp DNA mini kit (Qiagen) following the manufacturer's instructions. The extracted DNA was then amplified in simplex and multiplex polymerase chain reaction (PCR) assays using specific primers for *Salmonella enterica* (*hilA*) and *Shigella* species (*ipaH*), and subsequently, additional primer pairs specific for *Shigella sonnei* (*wbgZ*) and *Shigella flexneri* (*rfc*) were used (11).

The condition for PCR amplification was performed in a reaction mixture with total volume of 25µL, containing 2.5µL 10xTaq polymerase buffer, 0.3µL dNTPs (10mmol/L), 1U Taq DNA polymerase, 0.6µL $MgCl_2$ (50mmol/L) and 0.3 mol/L of each primer pair. The PCR procedure was as follows; initial denaturation step at 94°C for 5 minutes followed by 30 cycles, consisting of denaturation (94°C for 1 min), annealing (58°C *ipaH*, 65°C *hilA*, 60°C *wbgZ* and *rfc*) for 1 min, was set separately for each primer pair, and extension at 72°C for 1 min, followed by final extension step at 72°C for 5 min (11). Electrophoresis of the amplified products along with a molecular DNA marker was carried out on 1.5% agarose gel, visualization of the ethidium-stained gel was done in a UV transilluminator, and was photographed with a camera.

Widal antibody agglutination test

Only patients with positive Widal test (≥1:80) were recruited for the study. The Widal agglutination test was conducted to detect *Salmonella* agglutinin (antibody) from the serum of the patients. Briefly, the Widal test was performed with Cromatest kit as follows; serum samples were screened using the slide agglutination test which measures agglutinating antibodies against the lipopolysaccharide 'O' and protein flagellar 'H' antigens of *Salmonella* Typhi and *Salmonella* Paratyphi A and B. Serial dilution of sera starting at a dilution of 1:40 were made with 0.9% saline and examined for visible agglutination. Widal test was considered positive when a titre of ≥ 1:80 was observed according to routine laboratory procedures (12).

Table 1: Oligonucleotide primers used in this study (11)

Primer target		Primer sequence (5′ – 3′)	Amplicon size (bp)
ipaH	*ipaH-F*	GTTCCTTGACCGCCTTTCCGATACCGTC	619
	ipaH-R	GCCGGTCAGCCACCCTCTGAGAGTAC	
hilA	*hilA-F*	CGGAACGTTATTTGCGCCATGCTGAGGTAG	784
	hilA-R	GCATGGATCCCCGCCGGCGAGATTGTG	
Sflex-rfc	*Sflex-rfc-F*	TTTATGGCTTCTTTGTCGGC	537
	Sflex-rfc-R	CTGCGTGATCCGACCATG	
Sson-wbgZ	*Sson-wbgZ-F*	TCTGAATATGCCCTCTACGCT	430
	Sson-wbgZ-R	GACAGAGCCCGAAGA	

Antimicrobial susceptibility testing

Antimicrobial susceptibility test (AST) was performed in duplicate on each isolated bacterium using the disk diffusion method of Bauer et al., (13) against 10 selected antibiotics and the average diameter of inhibition zone for each isolate was taken, and interpreted as susceptible or resistant following the recommended guideline of the Clinical and Laboratory Standards Institute (13).

Statistical analysis

Data were presented using frequency distribution tables and descriptive statistics. The prevalence of each isolate was determined by dividing the number of isolates by the total number of all the isolates, expressed in percentages. The prevalence of infection caused by each isolate was also determined by dividing the number of each isolate recovered from the patients by the total number of patients (expressed in percentages).

Results:

Of the 40 patients with suspected typhoid fever by the Widal test, 9 yielded distinct growth of *Salmonella enterica* confirmed by PCR, giving a 22.5% rate, with *Salmonella enterica* serovar Typhi (*Salmonella* Typhi) as sole bacterium in 5 (12.5%) patients and co-infection with *Shigella* spp in 4 (10.0%) patients (Tables 2 and 3). All the 40 patients cultured positive for enteric bacteria, with a total of 52 PCR-confirmed isolates; *S. enterica* 9 (17.3%), *Shigella* spp 20 (38.5%), *Shigella flexneri* 9 (17.3%) and *Shigella sonnei* 14 (26.9%) (Table 3). The gel electrophoresis of the PCR amplicons of representative *Salmonella* and *Shigella* isolates on simplex and multiplex PCR amplifications are depicted in plates 1 and 2.

The antibiotic susceptibility test (AST) in Table 4 showed that all the enteric bacterial isolates were multi-drug resistant (resistant to ≥ 3 antibiotic classes), and all were resistant to ceftriaxone and pefloxacin. *Salmonella enterica* isolates were 33.3% resistant to amoxicillin, 44.4% to augmentin and gentamicin, 55.5% to tetracycline, and 100% to ceftriaxone, nitrofurantoin, ofloxacin, ciprofloxacin and pefloxacin. *Shigella* spp were 70% resistant to gentamicin and tetracycline, 80% to augmentin, 85% to amoxicillin, 90% to cotrimoxazole, 95% to nitrofurantoin, ofloxacin and ciprofloxacin, and 100% to ceftriaxone and pefloxacin. *Shigella flexneri* isolates were 55.5% resistant to gentamicin and tetracycline, 66.6% to augmentin, and 100% to amoxicillin, cotrimoxazole, nitrofurantoin, ceftriaxone, ofloxacin, ciprofloxacin and pefloxacin. *Shigella sonnei* isolates were 64.3% resistant to gentamicin and amoxicillin, 78.6% to tetracycline, 85.7% to augmentin, 92.9% to ofloxacin and ciprofloxacin, and 100% to cotrimoxazole, nitrofurantoin, ceftriaxone and pefloxacin.

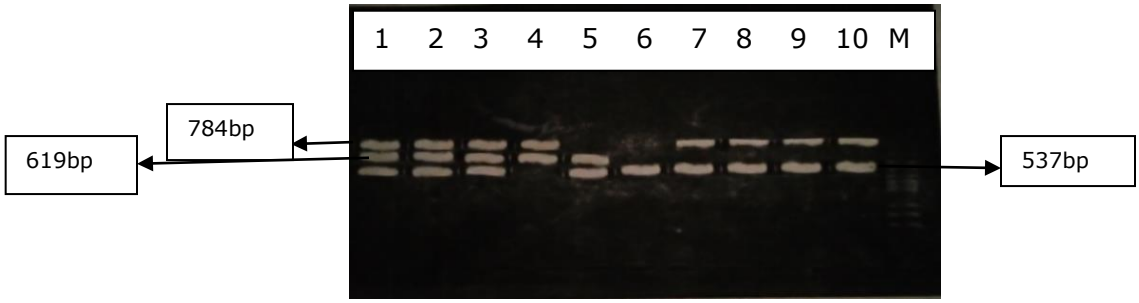
Table 2: Distribution of PCR-confirmed bacterial isolates from typhoid fever patients with positive Widal test

Laboratory code	Bacterial isolates	Frequency
1459	*Salmonella enterica*, *Shigella* spp	2
1460	*Salmonella enterica* serovar Typhi (*Salmonella* Typhi)	1
1461	*Salmonella enterica*, *Shigella* spp	2
1462	*Shigella* spp, *Shigella sonnei*	2
1463	*Shigella* spp	1
1464	*Shigella* spp, *Shigella flexneri*	2
1471	*Salmonella enterica* serovar Typhi (*Salmonella* Typhi)	1
1473	*Salmonella enterica*, *Shigella* spp	2
1474	*Shigella* spp, *Shigella sonnei*	2
1481	*Shigella flexneri*	1
1482	*Salmonella enterica* serovar Typhi (*Salmonella* Typhi)	1
1485	*Salmonella enterica* serovar Typhi (*Salmonella* Typhi)	1
1487	*Shigella flexneri*	1
1488	*Shigella* spp, *Shigella sonnei*	2
1489	*Shigella sonnei*	1
1566	*Shigella* spp	1
1569	*Shigella flexneri*	1
1570	*Shigella* spp	1
1571	*Shigella* spp, *Shigella sonnei*	2
1572	*Shigella* spp, *Shigella sonnei*	2
1574	*Shigella* spp	1
1578	*Shigella sonnei*	1
1579	*Shigella sonnei*	1
1580	*Salmonella enterica* serovar Typhi (*Salmonella* Typhi)	1
1581	*Shigella* spp, *Shigella sonnei*	2
1582	*Shigella* spp, *Shigella sonnei*	2
1637	*Shigella* spp	1
1639	*Shigella* spp	1
1641	*Shigella* spp	1
1642	*Shigella flexneri*	1
1643	*Shigella sonnei*	1
1644	*Shigella sonnei*	1
1645	*Shigella flexneri*	1
1646	*Shigella sonnei*	1
1647	*Shigella* spp	1
1667	*Shigella flexneri*	1
1668	*Salmonella enterica*, *Shigella sonnei*	2
1669	*Shigella flexneri*	1
1678	*Shigella* spp	1
1684	*Shigella flexneri*	1

Table 3: Distribution of enteric bacterial isolates by species

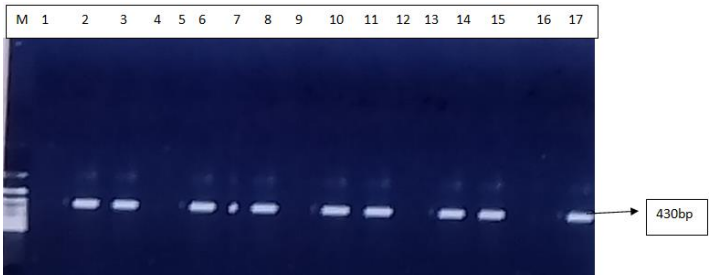
Bacterial isolate	Number of isolates (%) (n=52)	***Number of patients (%) (n=40)
Salmonella enterica	9 (17.3)*	9 (22.5)**
Shigella spp	20 (38.5)	20 (50.0)
Shigella flexneri	9 (17.3)	9 (22.5)
Shigella sonnei	14 (26.9)	14 (35.0)

* = 5 (9.6%) of 52 isolates (from blood) are *Salmonella enterica* serovar Typhi; ** = 5 (12.5%) of 40 patients had *Salmonella enterica* serovar Typhi isolated from their blood; *** = 12 patients had had two bacterial species while 28 patients had one bacterial specie isolated from their samples



Shigella species *ipaH* amplicon (molecular weight = 619bp), *Salmonella* species *hilA* amplicon (molecular weight = 784bp), *Shigella flexneri rfc* amplicon (molecular weight = 537bp)

Plate 1: Gel electrophoresis of multiplex PCR amplicon of representative *Salmonella* and *Shigella* isolates



Shigella sonnei wbgZ amplicon (molecular weight = 430bp)

Plate 2: Gel electrophoresis of PCR amplicon of representative *Shigella sonnei* isolates

Table 4: Antibiotic resistance profile in enteric bacterial isolates

Antibiotics	***Salmonella enterica* (n=9)**	***Shigella* species (n=20)**	***Shigella flexneri* (n=9)**	***Shigella sonnei* (n=14)**
Amoxicillin	3 (33.3)	17 (85.0)	9 (100.0)	9 (64.3)
Augmentin	4 (44.4)	16 (80.0)	6 (66.6)	12 (85.7)
Ceftriaxone	9 (100.0)	20 (100.0)	9 (100.0)	14 (100.0)
Nitrofurantoin	9 (100.0)	19 (95.0)	9 (100.0)	14 (100.0)
Gentamicin	4 (44.4)	14 (70.0)	5 (55.5)	9 (64.3)
Cotrimoxazole	8 (88.9)	18 (90.0)	9 (100.0)	14 (100.0)
Tetracycline	5 (55.5)	14 (70.0)	5 (55.5)	11 (78.6)
Ofloxacin	9 (100.0)	19 (95.0)	9 (100.0)	13 (92.9)
Ciprofloxacin	9 (100.0)	19 (95.0)	9 (100.0)	13 (92.9)
Pefloxacin	9 (100.0)	20 (100.0)	9 (100.0)	14 (100.0)

Discussion:

The importance of precise and timely diagnosis of typhoid fever in early stages of infection is crucial to identifying the etiological agent and carriers that might serve as source of transmission during outbreak (3), and this may subsequently avert inaccurate estimation of the global burden of this disease (2,14). In this study, a total of 52 PCR-confirmed bacterial isolates were recovered from blood and stool samples of 40 patients clinically suspected to have typhoid fever from positive Widal test; *Salmonella enterica* (17.3%, 9/52), *Shigella* spp (38.5%, 20/52), *Shigella flexneri* (17.3%, 9/52) and *Shigella sonnei* (26.9%, 14/52). Five of the *S. enterica* were recovered as sole isolates from blood cultures of only 5 (12.5%) of the 40 patients, and because only serovar Typhi frequently cause blood stream infection, these five *S. enterica* isolates are assumed to be *Salmonella* Typhi. This low *S.* Typhi isolation rate from patients considered to have typhoid fever by positive Widal test (≥1:80) and the high rate of isolation of other enteric pathogens (*Shigella* spp) aside *S.* Typhi emphasizes the low sensitivity and specificity (from possible cross-reactivity from other enteric pathogens) of the Widal test in the diagnosis of typhoid fever (5,15). Although, several studies have documented inherent variabilities of the Widal test, difficulty in establishing a steady state baseline titre, and lack of reproducibility of the test result (16,17,18), evidence from our study might suggest that a positive Widal test correlates more with infections caused by other enteric bacteria aside typhoid fever.

Shigella spp (especially *S. dysenteriae*) which was the most predominant bacterium in this study is known to cause inflammation of the large intestines, resulting in diarrhea stool containing blood or mucus. On the other hand, *S. sonnei* and *S. flexneri* are known for enterotoxin production (19). *Salmonella* Typhi, which had a low prevalence in this study, is known to be responsible annually for over 3.4 million typhoid fever cases with about 2 million deaths globally (9), and 681,316 deaths in Africa (20). Our study however showed that the true prevalence of the aetiological agents of enteric fever can only be known through appropriate laboratory diagnosis. Consequently, the true prevalence of typhoid fever in our study area was found to be approximately 12.5%, indicating that for every 100 patients who tested positive for Widal test, only 12 actually have the disease. Previous studies have reported comparable prevalence of 11.3%, 14.1% and 18.7% of culture-confirmed typhoid fever (21,22,23). In contrast to our findings, some previous studies reported higher typhoid fever prevalence of 22.1% to 55% among febrile patients in various settings in Africa and Asia from blood cultures (5,24,25,). Another factor to be considered with cultural method is the opportunity of determining the susceptibility of the isolated aetiological agents of enteric fever, which is not possible with the Widal test. This is particularly imperative now that the world is fighting the serious challenge of antimicrobial resistance (AMR) with attendant prolonged hospitalization and healthcare cost, treatment failure and increased mortality and morbidity (23,26-29). To avert such negative trend, it is thus necessary to know the resistance pattern of aetiological agents of enteric fever.

In our study, high antimicrobial resistance (AMR) rates were observed to selected antibiotics, ranging from 33.3% to 100%, with *Salmonella enterica* being the only enteric isolates showing > 50% sensitivity to augmentin (55.6%), gentamicin (55.6%) and amoxicillin (66.7%), while all other isolates exhibited resistant rates of >50% to all the antibiotics. Only gentamicin among all the selected antibiotics showed some *in vitro* inhibitory activity on *S. enterica* (55.6% sensitivity), *S. flexneri* (44.5% sensitivity), *S. sonnei* (35.7% sensitivity) and *Shigella* spp (30% sensitivity). Ceftriaxone and pefloxacin were the two antibiotics with 100% of the isolates resistant to them. Our findings are in agreement with previous studies from other sub-Saharan Africa which reported MDR rates of 50% and 52% among typhoid isolates in Ethiopia and Ghana respectively (30-32). Also, Ohanu et al., (23) reported high resistant rates (22.8%-100%) for typhoid isolates to several older and relatively newer antibiotics. These high antibiotic rates may have, among other possible causes, resulted from antibiotic selection pressure created by the overuse and misuse of antibiotics for treatment of typhoid fever that have been misdiagnosed from the wrong use of the Widal test.

Conclusion:

The results of our study showed that only 12.5% of typhoid fever cases diagnosed by the Widal test had *Salmonella* Typhi isolated from their blood cultures while *Salmonella enterica* and *Shigella* spp were isolated from stool samples of other cases, with all the bacterial isolates being multi-drug resistant. There is need to adopt culture techniques for

laboratory diagnosis of febrile illnesses in order to improve treatment regimen. The fact that antimicrobial susceptibility test can also be performed with culture technique could further guide antibiotic prescription and reduce the risk of emergence of resistant bacteria.

Contributions of authors:

POD, TBT and FJB conceptualized the study; POD drafted the first manuscript. TBT and FJB contributed to the design of the study and revised the final draft of the manuscript. BHT and AHA supervised sample collections by OQO and CMO. POD, TBT, BHT, AHA and FJB supervised laboratory analysis by OQO and CMO. Data analyses and interpretations were done by TBT. All the authors approved the final version of the manuscript

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Conflict of interest:

Authors declare no conflict of interest

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