ASSESSING RELIABILITY OF WIDAL TEST FOR TYPHOID FEVER CASE DETECTION AMONGST OUTPATIENTS ATTENDING TWO HOSPITALS IN KANO STATE, NIGERIA

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ABSTRACT

Background: Typhoid or enteric fever is caused by *Salmonella typhi* that cause salmonella food poisoning is acquired by ingesting food or water contaminated with faeces of infected humans or animals. The bacteria multiply and spread from intestines into the blood stream affecting many organs.

Aim: This study was to assess the *Salmonella typhi* infection prevalence among outpatients attending two hospitals in Kano State. Secondly, to compare the reliability of Widal test with the blood and stool culture for isolating *S. typhi* regarded as "gold standard". Thirdly, determine the co-occurrence of typhoid fever infection with anaemia.

Methodology: The sample population (n=200) comprised children (n=118) 3-9 years old and adults (n=82) were screened for typhoid fever. The highest serum dilution with agglutination was taken as positive antibody titre. The blood samples were inoculated into thioglycolate agar for seven days and thereafter sub-cultured in Mac Conkey agar for 18 - 24 hours at 37ºC. Pale yellow to near colourless colonies were identified as *Salmonella* spp. Anaemia was assessed using blood pack cell volume (PCV).

Results: Overall, the Widal test prevalence rate was 89 (44.5%) with male (n=123) having 50 (40.1%) and females (n=87) with 39 (30.7%). There was significant difference ($p < 0.05$) between *Salmonella* species isolated from males, 38 (30.9%) and females, 27 (35.1%), and no significant difference between the Widal test kit with 44 (44.0%) and 45 (45%) prevalence with 32 (72.72%) and 33 (73.33%) isolates, respectively. Among the Widal test positive (n=44), 16 (36.4%) had PCV below 30%.

Conclusion: This study had shown that Widal test is not definitive and incapable of differentiating active from past exposure to infection compared to isolating *S. typhi* in culture media. Sole reliance on Widal test for case detection and management should be done with caution considering the clinical prognosis of bacteraemia and anaemia may lead to death.

Key words: Anaemia, Blood samples, *Salmonella* species, Stool culture, Typhoid fever, Widal test.

INTRODUCTION

Typhoid fever or enteric fever is a bacterial infection caused by *Salmonella typhi*, which is widely recognized as a major public health problem in many developing countries (Onile and Odugbemi, 1987, Ogunbiyi, (1997). The disease emerged as an important infectious disease in the early 19th century.

It is endemic in the Indian subcontinent including Bangladesh, South-East and Far-East Asia, the Middle East, Africa, Central and South America where multidrug resistance have been reported (Ochiai *et al*., 2008; Jenkins and Gillespie, 2009). It is a systemic infection and is transmitted through the faecal oral route by the consumption of contaminated water and food, particularly raw or undercooked meat, poultry, eggs and milk (Johnson *et al.*, 2011). Once the bacteria have gained entry into the body, they multiply and spread from the intestines to the blood stream affecting many organs. The consequence of wider dissemination throughout the body may lead to clinical complication possibly involving toxin production by *S. typhi* in blood. This could result into toxic shock syndrome toxin (TSST) production as seen in the case of *Staphyloccocus aureus* infection (O'Brien *et al*., 2006). Another infection called paratyphoid fever had similar symptoms to fever but it is generally considered to be a milder disease caused by related strain, *Salmonella paratyhpi*.

The annual incidence of typhoid is estimated to be about 17 million cases worldwide (WHO, 2008). It is often encountered in developing countries including Nigeria where they constitute serious sources of morbidities and mortalities (Ibekwe *et al*., 2008). Large number of *Salmonella* must be ingested to produce gastroenteritis because many of the cells are rapidly eliminated from the gastrointestinal tract by defecating. Where the remaining bacteria multiply to sufficient number, the symptom of gastroenteritis appears within 8-48 hours after eating food contaminated with *S. typhi* . Almost 80% of cases and death occur in Asia (Abro *et al*., 2009). Following ingestion, there is an incubation period of about 10 – 14 days (Okafor *et al*., 2007). The patients could experience a sudden onset of abdominal pain accompanied with diarrhoea. The stool may occasionally contain mucus or blood.

Typhoid cannot usually be diagnosed from its symptoms alone, so a blood culture is a laboratory test to check bacteria in the blood sample. The Widal test is another blood test to screen for typhoid, however, it may not be very specific (WHO, 2003). Sometimes, the urine or stool of an infected person is checked for bacteria. A bone marrow test may also be performed though this is not commonly done. Other laboratory tests include a haemoglobin test, since typhoid can cause anaemia and liver function tests. Agglutination is a classic serologic reaction that results in clumping of a cell suspension by a specific antibody, directed against a specific antigen. The Widal test is a presumptive serological test for enteric fever or undulant fever. In case of *Salmonella* infections, it is a demonstration of agglutinating antibodies against O-somatic and H-flagellar antigens (Tanyigna *et al*., 1999) and the levels reflected severity of infection with *Salmonella* in the blood (Olopoenia *et al*., 2000). Generally and Nigeria in particular, the Widal agglutination test is about the sole laboratory diagnostic tool employed to buttress clinical diagnosis of enteric fever for the purpose of directing therapeutic measures specifically against this malady (Ibekwe *et al*., 2008). The *Salmonella typhi* usually need iron to sustain their intracellular growth and will rely on the red blood cells (RBC) which have iron as a central metal (iron chelated by four heme group). Low iron in the blood means low RBC counts, resulting in anaemia. Also implicated is the possibility of RBC's depression due to bloody diarrhea, since haemorrhage can occur in typhoid fever, most likely in the intestinal lining where most *Salmonella typhi* infection starts.

The aim of the study was to determine the prevalence of typhoid fever among outpatients (children and adults) attending two hospitals in Kano State. One of the specific objectives is to determine if the Widal test can differentiate between those with active infection from those with past exposure to infection using blood and stool culture methods for isolating *S. typhi* organisms from samples as "gold standard".

To assess the co-occurrence incidence of typhoid fever with anaemia in children attending hospitals to seek for medical care and diagnostic services.

MATERIALS AND METHODS

Study Area and population

Infectious Disease Hospital Kano (IDH) is a 200 bed hospital located at Sabon Gari Kano in northwest geopolitical zone of Nigeria with about 300 outpatients per day. Sabon Gari is cosmopolitan area within Kano Metropolis with diverse ethnic groups that cut across the country and neighbouring countries. Wudil General Hospital (WGH) in Wudil town is located in the south east of Kano, along Kano-Maiduguri road on latitude 11^0 $48^{,}$ N and the longitude 8^0 $51^{,}$ E. Wudil town is one of the important commercial towns in Kano State. Wudil LGA covers about 458km^2 and has approximately 105,106 inhabitants based on 2006 population census projected at 3.5% growth rate per annual. The Wudil River is used for irrigation of farms and cultivation of vegetables. Prevailing environment conditions with the habit of open defecation greatly enhance enteric disease transmission when water is contaminated with human faeces and vegetables or fruits are consumed unwashed or not properly washed. The study population targeted only outpatients that presented themselves at the hospitals complaining of fever, stomach and body pain seeking diagnostic service and treatment with request form. A total of 200 samples were collected over a period of December, 2016. The study population (n_0) was derived from the formula:

$$n_0 = \frac{Z^2pq}{C^2}$$ used to calculate the sample size for proportion

Where Z is 95% confidence level, p is estimated prevalence, q is 1-p, and C is the desired level of precision or margin of error at 5% or 0.05. The calculated sample size was 180.

Ethical Clearance

All human samples were obtained with free consent of participants under strict confidentiality of information. Ethical clearance approval was obtained from Hospital Management Board Ethical Committee, Ministry of Health, Kano State, Nigeria. Human blood samples were used only for the purpose of research work.

Blood Sample Collection

The blood and stool samples of children (below 10 years of age) and adults attending IDH and WGH were collected. A tourniquet was used to tie the arm to make the vein appear more prominent and 70% ethanol applied to disinfect the area. Venipuncture was performed using sterile disposable hypodermic syringe and needle to collect about 5 ml blood into ethylene diamine tetra acetic acid (EDTA) container. About 2ml of blood was used for Widal test and to determine the PCV according to Woo, (1970) and the remaining 3ml were stored in the refrigerator for media culturing.

Screening of Typhoid Infection Using Widal test

Two commercially available serodiagnostic Widal kit (ANTEC and Omega Diagnostic Products) febrile kit containing *S. typhi* antigens were used following the manufacturers' instructions. Briefly, a drop of the suspended antigen was added to an equal amount of serum. An initial positive screening test was performed in a checkerboard to determine the strength of the antibody. This was done by adding together equal volume of antigen suspension and serially diluted serum from the suspected patient. Agglutinations were visualized as clumps. Weakly reactive agglutinations were viewed under adequate light source, while strongly reactive agglutinations were easily visible. The results were scored as negative or 0 with no agglutination reaction and the serum that exhibits agglutination at and above ¼ dilutions was considered the end-point titre as described by Olopoenia *et al*. (2000).

Tube macroscopic agglutination test was performed for samples that showed weak agglutination in the rapid slide test (Gaultney *et al*., 1971).

The Widal test developed by Widal (1896) was performed by placing a drop of the serum on the slide, mixed together with the *Salmonella* O and H antigens and the type of agglutination as described by Cheesbrough, (2005) were observed as granular and more uneven type of clumping, respectively. According to Oloponia and King (2000) antibodies to these antigens are present in 6-8 days and 10-12 days, respectively. Following infection, there is a 4-fold rise in either of these antibodies between acute and convalescent sera is diagnostic (WHO, 2003). The test is only moderately specific for typhoid test.

Isolation and identification of *Salmonella*

The Widal test positive blood samples were inoculated into the thioglycolate agar dispensed in bottles and incubated at 37^0C for seven days. Thereafter, the blood in thioglycolate agar was subcultured into MacConkey agar and incubated for 24 hrs at 37ºC.

Collection of Stool Samples from adults

Stool samples collected from patients were processed within two hours and where there was a delay the specimens were transported to the laboratory in a cold box with ice packs and stored in a refrigerator at 4°C. Where a stool sample cannot be obtained, rectal swabs were inoculated into Carry Blair transport medium as described (Wain *et al*., 2001)**.**

Determination of Anaemia among children: Blood was drawn into a heparinized capillary tube leaving one third of it unfilled and one end of the capillary tube was sealed with plaster seal. The capillary tubes were centrifuged at 3000rpm for 5 minutes in microhaematocrit centrifuge and the packed cell volume (PCV) was measured on a haematocrit reader (Cheesbrough, 2005).

Gram Staining

Smear of about 20mm in diameter was made on a clear grease free slide, air dried and fixed by passing over a gentle flame 3 times, it was flooded with crystal violet solution for 1 minute. Excess crystal violet was decanted and slides were washed with distilled water in slanting position. Lugol iodine was added for 30 seconds, it was rinsed with water and then decolorized with acetone added on slide in slanting position for 10 seconds. The slide was rinsed with water and counter stained with neutral red for 1 minute and then examined under a microscope at 100x magnification (oil immersion) objective (Cheesbrough, 2010).

Biochemical Test

Isolates from colonies with growth characteristics of *Salmonella* were subjected to the following biochemical tests as described by WHO (2008).

Triple sugar iron agar test

The different groups or genera of enterobacteriaceae were differentiated based on capability to ferment glucose with the production of acid. It was based on the difference in carbohydrate fermentation pattern and hydrogen sulfide production by the various groups of intestinal organisms. Carbohydrate fermentation is indicated by the presence of gas and a visible color change of the pH indicator or phenol red. The production of hydrogen sulfide in medium was indicated by the formation of a black precipitate that blackened the medium in the butt of the tube (Cheesbrough, 2005).

Indole test

The test organism was inoculated into a test tube containing 3ml of peptone water and incubated at 37º C for 24 hours. A bout 0.5 ml of Kovac's reagent, which contained 1-p-dimethylamine benzaldehyde was added. The test tube was shaken gently and the development of rose pink coloration on the surface of the medium indicated a positive reaction of indole production within 10 minutes (Cheesbrough, 2010).

Methyl red – Voges Proskauer broth

Organism was grown in 5ml methyl red – Voges Proskauer (MR-VP) broth and incubated for 48 hours at 35ºC.

Thereafter, 1ml of the broth was transferred into a test tube and 2 drops of methyl red was added. Formation of red colour indicated positive methyl red test, a yellow colour indicated negative test.

Fifteen (15) drops of 5% alpha-naphthol in alcohol was added to the remaining broth and 5 drops of 40% KOH was also added and shaken. The cap of the tube was loosened and development of a red colour within 1 hour indicated a positive test (Cheesbrough, 2010).

Motility test using hanging drop slide

A pure culture of the organism was allowed to grow in Nutrient Broth (NB). One drop of cultured broth was placed on clean cover-slip, inverted over the concave depression of slide and Vaseline was applied around the concave depression for better attachment and to prevent evaporation of the fluid by air current. The slide was then examined carefully under high power objective (100x) immersion oil of a compound light microscope as described by Singh and Mcfeters, (1992). The swinging movements of bacteria were observed under contrasting background. The motile bacteria were identified as *Salmonella*.

Statistical Analysis

Observed differences in the analysis of variables between two groups were subjected to Chi-square (X^2 - test) and Student T-test of unequalled data. Analyses of differences among groups were subjected to multivariate analysis of variance (ANOVA) at 5% confidence interval (CI).

Conflict of Interest: The authors declared that there was no conflict of interest whatsoever.

RESULTS

Widal test positive: Overall result showed that 89 (44.5%) were Widal test positive, male (n=123) had 50 (40.7%) and female (n=77) with 39 (50.6%) prevalence. *Salmonella* species isolated from Widal test positive blood samples (n=45) with 33 (73.3%) and the gender distribution is as shown on Table 1. The difference between males and females was statistically significant ($p<0.05$) X^2-test.

Stool Culture test positive: Among the Widal test positives (n=89), *Salmonella* spp. were isolated from 65 (73.0%) in blood culture were the true positive (TP) and 24 (27.0%) were false positive (FP) cases. *Salmonella* species isolated from subpopulation (n=100) blood and stool culture were 33 and 32, respectively with 97% concordance.

Biochemical and morphological identity of isolates

All the bacteria organisms isolated from culture media were confirmed based on the biochemical IMVIC tests outcome. The Gram negatively stained, morphological rod shaped appearance and motility characteristics of *Salmonella species* were confirmed in 65 (73.0%) from the Widal test positive cases (n=89).

Table 1: Gender prevalence of salmonellosis in the Widal test positive samples

Sex	No. examined	No. positive (%)	
		Widal test	Cultured isolates
Male	123	50 (40.7)*	37 (30.1)β
Female	77	39 (50.7)*	28 (36.4)β
Total	200	89 (44.5%)	65 (32.5)

Differences between males and females were statistically significant for *Widal test and $^{\beta}$blood or stool test by X^2-test.

Gender and Age Analysis of Widal Test

The Widal test positive cases cut across all the age brackets irrespective of whether they are children or adults as shown on Table 2. Those aged ≥46 years (n=19) had the highest prevalence of 52.6% followed by 31-40 years with 46.7% and children <10 years old n=123 with 43%. The observed differences were not statistically significant (p>0.5) by ANOVA.

Table 2: Gender and Age Class Analysis of Widal Test Results

Age class in years	No. examined	No. of cases (%)		
		Male	Female	Total
<10	127	22 (17.3)	33 (26)	55 (43)
11-20	28	7 (25)	3 (10.7)	10 (35.7)
21-30	20	6 (30)	1 (50	7 (35)
31-40	15	4 (26.7)	3 (20)	7 (46.7)
>41	19	8 (42.1)	2 (10.5)	10 (52.6)
Total	**200**	**47 (23.5)**	**42 (21)**	**89 (44.5)**

Gender and Age Analysis of Anaemia in Tests Positive Children

Among the Widal test positive cases randomly selected (n=44), 16 (36.4%) had PCV below 35% ‘

(Table 3). The computed sample population mean ± standard deviation (STDEV) is 28.9±2.3; males, 28.3±3.0 and females, 29.1±2.0 with a range of 24-32%. The males had significantly higher number of anaemia (p<0.05) than the females using (X^2-test). In addition, prevalence of anaemia between the two age classes was not statistically significant (p>0.05) using X^2-test (Table 4).

Table 3: Gender analysis of anaemia in Widal test positives

Sex	Sample size, (%)	Culture positive (%)	Low PCV* (%)
Male	21 (47.7)	14 (66.7)α	9 (64.3)β
Female	23 (52.3)	18 (78.3)α	7 (38.9)β
Total	44 (100)	32 (72.7)	16 50.0)

Anaemia is regarded as PCV below standard range of 30-45% according to WHO, (2008) guideline.

Observed differences in $^{\alpha}$culture isolation of typhoid and $^{\beta}$co-occurrence of anaemia were both statistically significant by X^2-test.

Table 4: Age Analysis of Anaemia in Culture Test Positive Samples

Age Class	Sample size (n)	Widal test +ve (%)	Low PCV* (%)
3-6 years	47	18 (38.3)	7 (38.9)
7-9 years	53	26 (49.1)	9 (34.6)
Total	100	44 (44)	16 (36.4)

Anaemia is regarded as PCV below standard range of 30-45% according to WHO, (2008) guideline. Packed cell volume, .PCV, Percentage (%) and positive, +ve.

Socio-economic status of adult sample population

The sample subpopulation of those >10 years were classified into farmers (n=37), traders (n=40), housewives (n=16) and civil servants (n=7). The farmers had the highest infection, followed by traders, house wives and teachers have the least with 23 (52.3%), 10 (22.7%), 9 (20.5%), and 2 (4.5%) were Widal test positive cases, respectively.

DISCUSSION

Because the Widal tests are moderately specific for typhoid fever and relatively inexpensive when compared to bacterial culture methods they are still widely used (Bakr *et al*., 2011, Bhan *et al*., 2005, Bhutta, 2006, Parry *et al*., 2011). The test is possibly considered to be more of cost-benefit particularly in non-endemic settings (Chew *et al*., 1992) often without access to other diagnostic facilities. With the overall incidence of 44.5% as shown on Table 1 has shown that typhoid fever remained a common public health problem with children and women who are more vulnerable at risk of infections (Philip, 2000, Adeleke *et al*., 2006). There was no significant difference ($p>0.5$, X^2-test) between males and females in both Widal test and the two blood and or stool culture methods, which showed 97% concordance. Choice of blood sample culturing has advantage of detecting bacteraemia in addition to its slightly superior diagnostic reliability than stool culture. Bacteraemia can occur at any stage of the illness from the first 7-10 days and during relapse (Okafor *et al*., 2007) and portend serious complication of multiple organ dysfunctions, which can lead to death (Cheng *et al*., 2016).

Of more importance and worthy of note is the near concordance between blood and stool culture methods with 33 (75.0%) and 32 72.7%) given 97% concordance in isolating *Salmonella* species had proven the inability of Widal agglutination test to discriminate active infections from past exposure to infection. This further justifies the use of any one of the two culture methods which is a definitive diagnosis and gold standard to complement the screening test. A similar observation was made with 3(12%) *Salmonella* in both stool and blood (Eze *et al*., 2011) suggesting the presence of bacteria in stool is indicative of its presence in blood.

From the data presented on Table 2 showed that there was no gender difference between groups of males (23.5%) and females (21.0%) prevalence, respectively. The observed differences between the age group were not statistically significant by multivariate ANOVA ($p>0.05$). On Table 3 showed that higher infection rate in female children than males (40.7 and 50.7%) contradict the assertion by Alfred *et al*. (2005) that there may be genetic predispositions to typhoid fever in female since the finding found high incidence female children. This may contradict the claim that X-chromosomes confers some immunity to infections and females are doubly endowed (Dhawan and Dasai, 1996, Pinheiro *et al*., 2011) may not be true.

Anaemia is common in typhoid fever that could develop relatively, rapidly and are times profound. From data presented on Table 3, it can be inferred that the 36.4% co-occurrence of anaemia in patients (children, n=100, aged <10 years) with typhoid fever is in agreement with the findings of others. Anaemia in 52 (57%) of children <5 years of age with typhoid and para-typhoid infections in a holoendemic malarial area (Duggan and Beyer, 1975), malaria may have caused the concomitant anaemia.

The outcome of this study showed that 24.5% false positive cases among the Widal test positive cases (n=89) had further confirmed the unreliability of the test. Empirical evidence from this study did not support sole reliance on serological screening test for typhoid fever detection. In doing so, it should be an issue of serious concern for clinicians handling such cases to exercise high level of due diligence.

A corollary deduction from our finding was that not all infection may grow in culture for one reason or the other as enumerated. Sensitivity of blood cultures is between 30–70% depending on the volume of blood culture, presence of antibiotics (Gilman *et al*., 1975) and contaminated blood cultures can underestimate the burden of disease.

The sample population was drawn from outpatients attending hospitals seeking medical attention. Only field based surveillance could identify individuals are healthy carriers known in endemic areas with 35.9% apparently healthy persons have been detected with normal antibody titres of *Salmonella antigens* (Tanyigna *et al*., 1999). Such persons may not seek medical attention and will be spreading the disease by shedding bacteria into the environment as open defecation is a common practice.

All the observed cases of anaemia 16 (50%) (Table 4) cannot be equivocally attributed to *Salmonella* infection due to probable underlying malaria, which is also highly endemic in the study area. The co-occurrence of anaemia in salmonellosis buttressed the need for definitive diagnostic using blood culture to detect bacteraemia in addition to other clinical evaluation for effective case management. The subpopulation enlisted consisted of children <10 years, so pregnancy which is a factor among women who are naturally prone to anaemia was excluded. Secondly, this study did not evaluate the possible role co-infection with malaria plays in salmonellosis patients, which have been widely reported by Ammah *et al*. (1999); Alhassan *et al*. (2012); Igharo *et al*. (2012); Ukeagbu *et al*. (2014) among other research articles.

This study showed that socio-economic status influenced risk of infection as farmers had the highest numbers of Widal test positive rate (52.3%). This may be attributed to occupation associated hazard; which included drinking untreated water, eating fruits and vegetables that were not properly washed, using human faeces as manure, and working in environment or farm lands used for open defecation, etc. The traders (22.7%) and closely trailed by house wives (20.5%) both could have contracted infection from food consumption, while the civil servants were the least infected (2%) possibly haven the ability to afford drinking potable sachet table water. The bias in sample sizes portrayed low infection risk, which is inversely associated with tendency to seek for medical attention or hospital attendance. The socio-economic and environmental narratives that prevailed in the study area laid credence to Pinheiro *et al*. (2011) suggestion that typhoid fever spread can be reduced greatly by providing clean water and proper hygienic conditions.

CONCLUSION

The Widal test has been accepted as reliable screening technique for typhoid and paratyphoid fever in endemic area where facilities are poor. In conclusion, Widal test should not be used alone for case detection and management of typhoid fever in Nigeria (Onile and Odugbemi, 1987). The need to incorporate stool and blood culture in the routine diagnosis of typhoid cannot be overemphasized. Hence, population surveillance to screen and identify the group of healthy carriers within endemic communities using stool culture test becomes paramount public health consideration. In addition, treated sources of domestic and drinking water are very important in rural areas where these facilities are lacking. Enlightenment programs on basic rules of hygiene for semi urban and rural communities of economically poor nations should be encouraged so as to limit the transmission of faecal-orally transmitted infections. In addition, the need to implement rural water scheme and eradication of open defecation are of utmost importance.

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