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Full Length Research Paper

Baseline *Salmonella* agglutinin titres in apparently healthy freshmen in Awka, South Eastern, Nigeria

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This study reports the prevalence of typhoid fever caused by *Salmonella typhi* between genders. It further establishes the *Salmonella* titres that are not diagnostically significant but normal in the study population and the titre that could be used as presumptively diagnostic of typhoid fever. Blood samples were collected from 200 subjects; 82 males and 118 females; and examined for the presence and levels of *Salmonella* antibodies by Widal agglutination technique. Standard *S. typhi* and *Salmonella paratyphi* "O" and "H" suspension (ANTEC) were used as antigens. Of the 200 sera tested, agglutinins to *S. typhi* were most prevalent in male subjects accounting for 39% of the "O" antigens and 41.5% of "H" antigens at the various dilutions while in the 118 female subjects, 10.7% accounts for the "O" and 29.5% for the "H" antigens. There was a male preponderance (M/F 2:1). Since the positive sera with titres of ≥ 80 occurred in more than 5% of the samples, this study therefore suggests that such titres be regarded as normal among the communities studied while there should be a high index of suspicion of clinical infections in titres above 80 when a second serum is impractical. This will improve accurate diagnosis. Improving accurate diagnosis is the surest way to reverse the deteriorating health status of Nigerians.

Key words: Diagnosis, gold standard, public health, typhoid fever, widal test.

INTRODUCTION

Salmonella typhi is a bacterium that causes typhoid fever (enteric fever). Typhoid fever is an acute, life-threatening febrile illness caused by the bacterium *Salmonella enterica* serotype Typhi (CDC, 2008). Typhoid fever is a global infection with a fatality rate of 10%. The disease is a cause for concern and a major public health problem in developing countries (Asia, Africa), especially in Nigeria due to poor sanitary conditions and lack of or inadequate potable water (Anita et al., 2002; Doughari, 2005). The World Health Organization (WHO) estimated an annual infectious rate of 21.6 million and approximate death rate of 600 000 with the highest percentage in Africa and Asia (WHO, 2008).

Typhoid (enteric) fever caused by *S. typhi* is an endemic disease in the tropic and sub-tropic and has become a major public health problem in developing countries of the world with an estimated annual incidence of 540 per 100,000. It is often encountered in tropical countries including Nigeria where they constitute serious sources of morbidities and mortalities. It is caused by *S. typhi*. Gram negative bacteria which are motile, though non-flagellate variants occur. Capsules are not formed. They are intestinal pathogens which comprises a species *S. typhi* which causes an enteric fever known as typhoid fever (Philip, 2000). *S. typhi* has somatic antigens and glycolipid microcapsule the VI or virulence antigen. Phage typing can distinguish different strains of the organism. Enteric fever caused by *S. typhi* is often en-

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countered in tropical countries including Nigeria where they constitute serious sources of morbidities and mortalities (Baver, 1973). It is a major public health problem in the developing countries of the world with an estimated annual incidence of 540 per 100,000 (Abuobeida, 1996). An estimated 22 million cases of typhoid fever and 200,000 related deaths occur worldwide each year (Crump et al., 2004). Approximately 400 cases of typhoid fever among persons with onset of illness in the United States, most of whom are recent travelers, are reported to CDC each year (CDC, 2008).

Typhoid fever is transmitted by the faeco-oral route through food or water, contaminated with urine or faeces of a patient or a chronic carrier. It is transmitted through the ingestion of food or drink contaminated by the faeces or urine of infected people (CDC, 2008). Persons with typhoid fever carry the bacteria in their bloodstream and intestinal tract and can spread the infection directly to other people by contaminating food or water (Utah, 2005). Risk is greatest for travelers to South Asia and developing countries in Asia, Africa, the Caribbean, and Central and South America. Travelers to South Asia are at highest risk for infections that are nalidixic acid-resistant or multidrug-resistant (that is, resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole) (Ackers et al., 2000). Travelers who are visiting relatives or friends and who may be less likely to eat only safe foods (cooked and served hot) and beverages (carbonated beverages or those made from water that has been boiled) are at greater risk. Travelers have acquired typhoid fever even during brief visits of less than 1 week to countries where the disease is endemic (Steinberg et al., 2004).

S. typhi like all *Salmonella* is entirely parasitic. It differs from many of the others in that man is its only natural host and that even in the laboratory it is of low virulence for mice and other animals. Although epidemics are usually spread through water supplies or food, the source of the organism is always a human patient or carrier. Symptoms usually develop 1–3 weeks after exposure, and may be mild or severe. They include high fever, malaise, headache, constipation or diarrhoea, rose-coloured spots on the chest, and enlarged spleen and liver. Healthy carrier state may follow acute illness. The hallmark of typhoid infection is persistent, high fever as high as 103 to 104°C F (39 to 40°C). Other common symptoms and signs include headache, malaise, anorexia, splenomegaly, a rash of flat, rose-colored spots, and relative bradycardia (Parry et al., 2002). Many mild and atypical infections occur (CDC, 2008). Typhoid fever can be treated with antibiotics. However, resistance to common antimicrobials is widespread. Healthy carriers should be excluded from handling food (CDC, 2008).

The gold standard for its diagnosis rests on the recovery and identification of the causal organisms, from blood during the first few days of the illness, or from faeces during the second and third weeks of the illness or from urine during the third and fourth week (Mgbor and Osuafor, 1990; Easmon, 2002). A blood or stool sample is needed to diagnose typhoid fever. The samples are examined for *S. typhi* bacteria (Utah, 2005). The serological test, Widal test, is a well known test, used as an indirect test to detect the “shadows” “footprints” of *S. typhi* groups. The possibility of a quick serodiagnostic test for typhoid fever has engaged the attention of scientists in the last few years (Onunkwo et al*.,* 2001). Haemagglutination, coagulation, fluorescent antibody, enzyme linked immunosorbent assay (ELISA) and counter immunoelectrophoresis (CIEP) have all been used for the Widal serological diagnosis of typhoid fever (Abuobeida, 1996).

Recently, another promising approach in the diagnosis of typhoid fever involves the development of a method to detect IgM antibodies to *S. typhi* as an indicator of recent or on-going infection. According to Ibekwe (2004) a simple and sensitive adherence test to detect IgM antibodies to typhoid fever has been developed, which revealed that 95% of 61 sera from confirmed cases of typhoid fever (culture positive) possessed IgM antibodies to the “H” and/or “O” antigens of *S. typhi* while those with non-typhoidal fever common in the region (e.g. dengue fever, typhus, leptospiorosis) showed little or no reactivity in this test. A high “O” and low “H” agglutinins titre therefore suggest an active infection, whereas a low “O” and a high “H” suggest an anamnestic reaction. It is important to state that in Widal test, the first serum sample should be drawn on the first day of admission, and a second sampling is required one week later thus a rising titre over a 7-10 days period is necessary for a firmer diagnosis. The serological techniques can be performed either by the rapid slide agglutination test or by the tube dilution agglutination test (Widal test).

In Nigeria, 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 (Mgbor and Osuafor, 1990). As is generally known, the results of this serological test only become reliable if at least two properly staggered tests show about four-fold rise in antibody levels (Gilles, 1975). However, the scientific truism remains that only the bacteriological isolation of enteric fever bacteria from the patients’ blood, faeces or urine constitute unequivocal evidence of the infection (Opara and Nweke, 1991).

Also, in Nigeria, the facilities for this bacteriological isolation and identification of the typhoid organism in most hospitals are lacking (Onunkwo et al., 2001). Though, the Federal Ministry of Health has set up the tone for a mechanism that will reduce the errors emanating from faulty equipment, and substandard reagents Where the reverse is the case, this cultural option is prioritized downwards in favor of Widal serology for reasons of economy and diagnostic speed. This will ensure that doctors must not give medications before diagnosis.

The gold standard for the treatment of typhoid fever is choramphenicol (Easmon, 2002). Specific antimicrobial therapy shortens the clinical course of typhoid fever and reduces the risk of death. Persons who may have been exposed to *Salmonella enterica* serotype Typhi and who develop symptoms of typhoid fever should seek medical care. Antimicrobial therapy should be guided by data on antimicrobial sensitivity, particularly for travelers to South Asia. Patients should be monitored to ensure that fever wanes within a few days of starting treatment. If fever does not subside, alternative antimicrobial agents or other foci of infection should be considered (CDC, 2008).

However, ingestion with *S. typhi*, *Salmonella paratyphi* and *Salmonella schottmulerri* confers a certain degree of immunity. Reinfection may occur but is often milder. Circulating antibodies to "O" or "Vi" are related to resistance to infection and disease. Though, relapses may occur in 2-3 weeks after recovery in spite of antibodies. Secretory IgA antibodies may present attachment of Salmonellae to intestinal epithelium.

Typhoid vaccination is not required for international travel, but CDC recommends it for travelers to areas where there is a recognized risk of exposure to *S. Typhi*. Vaccination is particularly recommended for those who will be traveling in smaller cities, villages, and rural areas off the usual tourist itineraries, where food and beverage choices may be more limited. While immunization is recommended, travelers should be cautioned that none of the available typhoid vaccines is 100% effective, nor do they provide cross-protection against other common causes of gastrointestinal infections. Typhoid vaccination is not a substitute for careful selection of food and drink (CDC, 2008).

This study reports the prevalence of typhoid fever caused by *S. typhi* between genders. It also further establishes the *S. typhi* titres that are not diagnostically significant but normal in the study population and the titre that could be used as presumptively diagnostic of typhoid fever.

MATERIAL AND METHODS

Sample collection

Blood samples of 200 freshmen (first year students) were collected at the University Health Centre of Nnamdi Azikwe University, Akwa, Anambra State, Nigeria; 118 females and 82 males. Two (2) milliliters of the blood samples were centrifuged at a high speed for 5 min in order to separate the serum from the blood cells.

Widal agglutination test

ANTEC febrile antigen kit (United Kingdom) was used for the Widal test. The rapid slide screening test was first carried out, followed by the tube agglutination test according to the manufacturer's specifications. The ANTEC febrile antigens are suitable for both the rapid slide and tube agglutination tests against human sera for the detection of these agglutinins. The stained antigen suspensions are killed bacteria, stained to enhance the reading of agglutination tests. The blue stained antigens are specific to the somatic "O" antigens whilst the red stained antigens are specific to the flagellar "H" antigens.

Rapid slide titration

Using a pipette, 0.08, 0.04, 0.02, 0.01 and 0.005 ml of undiluted serum was dispensed onto a row of 3 cm diameter circles. The reagent bottle was rigorously shaken and a drop of the undiluted antigen suspension was added to each serum aliquot. This was thoroughly mixed with the aid of a stirring stick and the slide was gently rotated. The reactions were observed after a minute. The agglutination observed in any circle was indicative of the following results in a test tube. 0.08 ml = 1:20, 0.04 ml = 1:40, 0.02 ml = 1:80, 0.01 ml = 1:160 and 0.005 ml = 1:320 (Cheesbrough, 2002).

Tube agglutination test

The positive results obtained through the rapid slide test were confirmed using the following techniques. Eight (8) small plastic tubes were labeled accordingly in a rack; with the aid of a pipette 1.9 ml of 0.85% saline was dispensed into the first tube, and 1 ml into the remaining seven tubes. 0.1 ml of the subject's undiluted serum was dispensed into the first tube. This was thoroughly mixed using a larger pipette volume and tip (i.e. set to 0.1 ml). 1 ml was pipetted from the first tube and then dispensed into the second tube. This was thoroughly mixed. This doubling dilution technique was continued serially up to the seventh tube. 1 ml from the seventh tube was discarded. The eight tubes contained only saline as a control and therefore did not contain any serum. The reagent bottle was shaken vigorously and a drop of the appropriate antigen suspension was added into each tube, thoroughly mixed and incubated appropriately as follows: *Salmonella* 'O' Antigens and proteus = 50°C for 4 h; *Salmonella* 'H' Antigen = 50°C for 2 h; *Brucella* Antigens = 37°C for 4 h and Typhi Vi = 37°C for 4 h. It was left overnight in the fridge, and then was allowed to reach room temperature before the tubes were read. The tubes were placed in a water bath up to 2/3rd the way up to the level of the tube content. This was in order to maintain convection current within the tube and thereby obviate false results. The tubes were examined after the appropriate incubation time and agglutination was checked for. The titre that was taken was the last tube to show agglutination (Cheesbrough, 2002).

RESULTS AND DISCUSSION

The distribution of *Salmonella* agglutinin titres obtained from 200 (100%) apparently healthy freshmen normal sera; 118 (59%) females and 82 (41%) males is shown in Tables 1 and 2. Table 1 shows the distribution of *Salmonella* agglutinin titres in 82 apparently normal male freshmen. Forty-two (51.2%) males had *Salmonella paratyphi* A-H agglutinin titre while 32 (39%) males had *Salmonella paratyphi* C-O, *Salmonella paratyhi* C-O, and *Salmonella paratyphi* C-H agglutinin titre respectively. Only 12 (14.6%) males had *Salmonella paratyphi* B-O agglutinin titres. The distribution of *Salmonella* agglutinin titres in 118 apparently normal female freshmen is shown in Table 2. Thirty-six (32.6%) of the females subjects used in this study had Salmonella paratyphi B-O agglutinin titre while only 10 (8.9%) had *S. paratyphi* C-H agglutinin titre (Table 2).

Table 1. Distribution of *Salmonella* agglutinin titres in 82 apparently normal male freshmen.

Salmonellae	Total No. Tested	No. not Titrated (%)	No. Positive (%)
S. paratyphi A-O	81	1	21 (25.6)
S. paratyphi B-O	79	3	12 (14.6)
S. paratyphi C-O	78	4	32 (39.0)
S. typhi O	80	2	32 (39.0)
S. paratyphi A-H	81	1	42 (51.2)
S. paratyphi B-H	80	2	19 (23.2)
S. paratyphi C-H	79	3	32 (39.0)
S. typhi H	80	2	34 (41.5)

Table 2. Distribution of *Salmonella* agglutinin titres in 118 apparently normal female freshmen.

Salmonellae	Total No. Tested	No. not Titrated (%)	No. Positive (%)
S. paratyphi A-O	116	2	14 (12.5)
S. paratyphi B-O	115	3	36 (32.6)
S. paratyphi C-O	117	1	28 (25.0)
S. typhi O	117	1	12 (10.7)
S. paratyphi A-H	116	2	19 (17.0)
S. paratyphi B-H	115	3	23 (20.5)
S. paratyphi C-H	116	2	10 (8.9)
S. typhi H	114	4	33 (29.5)

Table 3 depicts the number and percentage of sera with end titres in 82 apparently normal male freshmen. It was observed that with exception of *S. paratyphi* B-O and *S. paratyphi* B-H, all other agglutinins tested were present in the sera of the apparently normal males up to the titre of 160 and at frequencies ranging from 1.2-6.1% (Table 3).

However, the frequency of agglutinins of 80 ranged from 1.2 - 7.3%. It would seem that titres from 20-80 occurred in a significant proportion of the samples as shown in Table 3.

The number and percentage of sera with end titres in 118 apparently normal female freshmen is shown in Table 4. All agglutinins tested were present in the sera of apparently normal females up to the titre of 160 at frequencies ranging from 0.9-3.6%. For agglutinin of 80, the frequency ranged from 0.9 - 6.3% (Table 4).

In this study, the titres of *Salmonella* "H" in males were higher than those of the "O" whereas in the females, *Salmonella* "O" titres were higher than those of 'H'. Agglutinins to *S. typhi* were the most prevalent among the sera tested at various dilutions in the males, amounting to 39% for the "O" and 41.5% for the "H" than in the females with 10.7% for the "O" and 29.5% for the 'H'. Agglutinin level for the typhoid and paratyphoid group tested in this study were evidently very frequently found in the blood of the subjects. The levels of agglutinin of *S. paratyphi* B-O and *S. typhi* B-H in the males were however, very low. Agglutinin titres of 80 were observed in only 7.3 and 1.2% for *S. paratyphi* B-O and *S. paratyphi* B-H respectively. In the females, the agglutinin titres for *S. paratyphi* B-O and *S. paratyphi* B-H reached 160 for 3.6 and 1.8% of the sera, respectively.

In a situation where second sample collection is not feasible, knowledge of the agglutinin levels in the sera of normal subjects from the patients' community can form the baseline on which a diagnosis can be made (Opara and Nweke, 1991). For practical purposes, titres occuring in more than 5% of the subjects under study were not diagnostically significant and should be regarded as normal in that population (Collard et al., 1959). Based on this premise, it would seem that *Salmonella* titres of 80 occurred in significant proportion of the sample (1.2-7.3%) and (0.9-6.3%) in the males and females, respectively. Titres above 80 occurred in more than 3.7 and 3.6% of the male and female samples, respectively. Therefore, titres above 80 could be used in the presumptive diagnosis of enteric fevers in the study area, but should be confirmed if a second sample is possible.

The clinical history of the subjects was not documented. However, the high titre of antibodies unrelated to antigenic stimulation, or to infection with cross-reacting organisms other than *Salmonella*. For instance, cross reaction between a normally harmless gut bacterium *Citrobacter freundi* can confuse the diagnosis of typhoid bacilli sharing antigenic properties (Onunkwo et al., 2001). Moreover, a number of coliform bacteria for bacilli sharing antigenic properties (Onunkwo et al., 2001).

Table 3. Number and percentage of sera with end titres in 82 apparently normal male freshmen.

Salmonellae	No. Positive (%)	End titres					
		< 20	20	40	80	160	320
S. paratyphi A-O	21 (25.6)	2 (2.4)	7 (8.5)	3 (2.4)	6 (3.7)	2 (2.4)	1 (1.2)
S. paratyphi B-O	12 (14.6)	2 (2.4)	6 (7.3)	2 (2.4)	2 (2.4)	-	-
S. paratyphi C-O	32 (39.0)	6 (7.3)	10 (12.2)	8 (9.8)	6 (7.3)	2 (2.4)	-
S. typhi O	32 (39.0)	4 (4.9)	12 (14.6)	8 (9.8)	2 (2.4)	3 (3.7)	3 (3.7)
S. paratyphi A-H	42 (51.2)	4 (4.9)	20 (24.4)	9(11.0)	4 (4.9)	3 (3.7)	2 (2.4)
S. paratyphi B-H	19 (23.2)	2 (2.4)	10 (12.2)	6 (7.3)	1 (1.2)	-	-
S. paratyphi C-H	32 (39.0)	2 (2.4)	13 (16.0)	6 (7.3)	5 (6.1)	3 (3.7)	3 (3.7)
S. typhi H	34 (41.5)	8 (9.8)	10 (12.2)	9(11.0)	6 (7.3)	1 (1.2)	-

Table 4. Number and percentage of sera with end titres in 118 apparently normal female freshmen.

Salmonellae	No. Positive (%)	End titres					
		< 20	20	40	80	160	320
S. paratyphi A-O	14 (12.5)	2 (1.8)	3 (2.7)	3 (2.7)	2 (1.8)	2 (1.8)	2 (1.8)
S. paratyphi B-O	36 (32.6)	4 (3.6)	12 (10.7)	9 (8.0)	7 (6.3)	4 (3.6)	-
S. paratyphi C-O	28 (25.0)	3 (2.7)	8 (7.1)	7 (6.3)	5(4.5)	3 (2.7)	2 (1.8)
S. typhi O	12 (10.7)	1 (0.9)	3 (2.7)	3 (2.7)	2 (1.8)	2 (1.8)	1 (0.9)
S. paratyphi A-H	19 (17.0)	2 (1.8)	5 (4.5)	5(4.5)	4 (3.6)	2 (1.8)	1 (0.9)
S. paratyphi B-H	23 (20.5)	1 (0.9)	10 (8.9)	5(4.5)	5(4.5)	2 (1.8)	-
S. paratyphi C-H	10 (8.9)	1 (0.9)	4 (3.6)	2 (1.8)	1 (0.9)	1 (0.9)	1 (0.9)
S. typhi H	33 (29.5)	5 (4.5)	12 (10.7)	8 (7.1)	4 (3.6)	2 (1.8)	2 (1.8)

Moreover, a number of coliform bacteria for share antigens with *Salmonella*. This could lead to confusion in the serological diagnosis of typhoid fever. Therefore, serological findings have to be interpreted with a lot of caution particularly in country like Nigeria where there are yet to be laid down standard baseline titres (Ibekwe, 2004).

It is clear that *Salmonella* agglutinins are common among apparently healthy people. Titres beyond 80 should be an index of presumptive diagnosis of typhoid fever. Poor diagnosis leads to emergence of resistant strains of diseases. Efforts must be made however, to confirm the diagnosis by paired sera investigation more than in presently the case. Doctors should be more meticulous in their clinical assessment of patients before requesting Widal tests. Widal kits should be made available for epidemiological surveillance and survey in our medical centres. Therefore, more studies should be carried out to determine *Salmonella* agglutinin titres in the entire apparently healthy populations so that better judgment based on the prevailing agglutinin titres could be made.

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