Molecular Identification of Salmonella enterica serovar typhi from Stool Culture of Widal Test Positive Patients in Selected Hospitals in Abeokuta, Nigeria

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Abstract: Typhoid fever, a systemic disease caused by Salmonella enterica serovar typhi (S. typhi) remains a major public health problem in many parts of the world. Widal test appears to be the only laboratory means employed in the diagnosis of typhoid fever among suspected patients and the test continues to suffer from serious cross-reactivity with other infectious agents. This project was designed to confirm the diagnosis of typhoid fever from stool culture of Widal test positive patients using molecular method. Prospective study on stool samples of Widal test positive patients was conducted. Patients with Widal test results of titre value ≥ 1:80 were enrolled. Socio-demographic data of the respondents were obtained with open-ended questionnaire. Stool samples were collected and cultured from a total of 200 patients for isolation of Salmonella enterica serovar typhi. Isolates were identified and confirmed using cultural, morphological and biochemical tests. Further identification was done by molecular method using 16S rRNA gene amplification and sequencing. DNA sequences obtained were used for similarity search by Basic Local Alignment Search Tools (BLAST) on National Centre for Biotechnology Information (NCBI) database to identify the isolates molecularly. Phylogenetic tree of relationship among isolates and those on NCBI database was drawn using Molecular Evolutionary Genetic Analysis (MEGA) version 5.0. Antimicrobial susceptibility test for the isolates was determined using disc diffusion method. Descriptive statistics were computed for data analysis using Statistical Package for the Social Sciences (SPSS) version 17.0. Seven (7) isolates of Salmonella enterica serovar typhi was cultured from the 200 stool samples given a prevalence of 3.5 %. Five (71.4%) of the positive samples were isolated from patients age 0-18 while 2 (28.6%) were isolated from patients above 18 years. Also, 2(28.6%) of the isolates were from male and 5(71.4%) from female. The phylogenic analysis showed that six of the isolates formed separate clusters with the isolates from NCBI database. The isolates showed 90% sensitivity to ciprofloxacin, 80% to ofloxacin and 60% to sparfloxacin and pefloxacin. All the isolates were resistant to azithromycin and ceftazidine while 80% of the isolates were resistant to both augumentin and gentamycin. This study demonstrated that stool culture and molecular identification of Salmonella enterica serovar typhi should be part of standard methods for the diagnosis of typhoid fever in humans.

Key Words: Molecular Identification, Nigeria, Salmonella enterica, Stool Culture, Typhoid, Widal Test.

1. INTRODUCTION

Typhoid fever, a systemic disease caused by *Salmonella enteric serovar typhi (S. typhi),* remains a major public health problem in many parts of the world (Agada *et al.*, 2014). The bacillus is transmitted by milk, water, or solid food contaminated by faeces of typhoid victims or of carriers, that is, healthy persons who harbour typhoid bacilli without presenting symptoms. The incubation period of typhoid fever usually lasts one to three weeks (Shimoni *et al*., 1999).

The bacteria collect in the small intestine, from where they enter the bloodstream. Symptoms usually include headache, nausea, vomiting, fatigue, gastroenteritis, abdominal cramps and bloody diarrhea with mucus and sometimes reactive arthritis (Casmir *et al*., 2014). The disease spontaneously subsides after several weeks in most instances, but in about 20 percent of untreated cases the disease progresses to pneumonia, intestinal hemorrhage, and even death (Agada *et al.*, 2014).

Deaths from typhoid fever were greatly reduced by the isolation of the first antibiotic effective against the typhoid bacillus chloramphenicol, derived from a South American mold in the late 1940s. Because of widespread resistance to

chloramphenicol, antibiotics from the fluoroquinolone and cephalosporin groups, such as ciprofloxacin and ceftriaxone are currently the drugs of choice in the treatment of typhoid (Adachi *et al*., 2005).

The importance of typhoid fever in public health sector is a growing concern day by day throughout the world and over the last several decades, there have been significant shift in predominant *Salmonella* serovars associated with human infections (Adeleke *et al.,* 2006). Typhoid fever in the past has caused tremendous loss to society in many countries around the world (Stamer *et al.,* 2004).

Widal, a serological diagnosis test for enteric fever was founded in 1896 by Georges Fernand Isidore Widal. The role of Widal test has been to increase the index of suspicions for the presence of typhoid fever by demonstrating a positive agglutination. Unfortunately, in most developing countries including Nigeria the situation is quite different as the Widal test appears to be the only laboratory means employed in the diagnosis of typhoid fever among suspected patients and the test continues to suffer from serious cross-reactivity with other infectious agents that may produces false-positive results (Olopoenia *et al.,* 2000).

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 (Ibekwe *et al.,* 2008). 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 (Ibekwe *et al.,* 2008). While performance of the test may require some detailed technical work, interpreting the test result is the more arduous task (Olopoenia *et al.,* 2000). Since the ultimate goal of the test is antigen-antibody complex reaction, cross-reactions are encountered when antibody produced by nontyphoidal antigens reacts with typhoid-specific antigens (Ibekwe *et al.,* 2008).

Several other diseases caused by non-*Salmonella* organisms (malaria, tuberculosis, endocarditis, chronic liver disease, brucellosis, sepsis with other bacterial pathogens, tularaemia, leptospirosis, rickettsial diseases and viral infections such as dengue fever, acute hepatitis, and infectious mononucleosis) have been shown to exhibit this cross-reactivity in typhoid endemic regions (Maurice *et al.,* 2012). These cross-reactions increase the error rate of the result of the Widal test (Maurice *et al.,* 2012).

In addition, it is not a very accurate method, since patients are often exposed to other bacteria (e.g. *Salmonella enteritidis*, *Salmonella typhi*murium) that induce crossreactivity. Many people also have antibodies against these enteric pathogens, which also react with the antigens in the Widal test, causing false-positive results. Test results need to be interpreted carefully in the light of past history of enteric fever, typhoid vaccination, and general level of antibodies in the populations in the endemic areas of the world (Olopoenia *et al*., 2000).

Typhoid fever has an important socio-economic impact, so accurate diagnosis of the disease at an early stage is important not for etiological diagnosis but also for identifying individuals that may serve as potential carrier who may be responsible for acute typhoid fever outbreak (Zailani, *et al.,*2004). Due to the inexperience of some clinicians in typhoid endemic countries, many cases of pyrexia of unknown origin receive the diagnosis of typhoid fever, based upon a false-positive Widal test result rather than a positive culture of S. *typhi* (Olopoenia *et al*., 2000).

PROBLEM STATEMENT: In Nigeria, the Widal agglutination test is about the sole laboratory diagnostic tool employed to buttress clinical diagnosis of enteric fever and the titre value $\geq$ 1:80 is usually used as significant titre value in many parts of Nigeria to confirm typhoid fever in patients (Adeyi *et al.,* 2010).This titre value is not diagnostic enough to confirm typhoid fever (Smith *et al.,* 2004). There is therefore the need to confirm the diagnosis of *Salmonella enterica serovar typhi* from stool culture using molecular method.

2. METHODS

This is a prospective study of patients coming for check up at the Community Clinic of The Federal Neuropsychiatric Hospital, Aro, Abeokuta and Bisted Hospital, Abeokuta, Ogun State, Nigeria.

2.1. Widal Test : Qualitative slide agglutination and semi quantitative tube agglutination (titration) were performed using febrile antigen kits of *Salmonella typhi* (Chromatest Febrile Antigens kits, Linear chemicals, Barcelona, Spain). An antibody titre of 1:80 and higher were taken as cut off values to indicate recent infection of typhoid fever. Data and Stool samples were then collected after informed consent was obtained from each volunteer and guardian.

2.2.Culture of the Stool Samples : For the isolation and identification of *Salmonella enterica serovar typhi* from the specimens, standard conventional methods were used as described by Henry *et al*., (2012).

2.3. Molecular Characterization of *Salmonella enterica serovar typhi* : Molecular characterization of *Salmonella enterica serovar typhi*, sequence editing and database matching methods were used as described by Reyes *et al*., (2012) and Segata *et al.*, (2013).

2.4.Phylogenetic Analysis : Phylogenetic and molecular evolutionary relatedness of the bacterial isolates as well as homologous sequences retrieved from the NCBI database were conducted in MEGA version 5.0. After multiple sequence alignment and editing, a maximum likelihood tree was constructed using the Tamura-Nei substitution model (Tamura *et al.*, 2011).

2.5.**Susceptibility Tests for *Salmonella enterica serovar typhi* :** The susceptibility of *Salmonella enterica serovar typhi* to selected commonly prescribed antibiotics was performed using Kirby Bauer disc diffusion method (Cheesbrough, 2006).

3. RESULTS

All the isolates were confirmed as *Salmonella enterica serovar typhi* by molecular method using 16S rRNA gene amplification and sequencing (Table 1).

Table 1: Molecular identification of Isolated *Salmonella enterica serovar typhi*

Isolates	Hospitals	Location of Patients	Sequence Length	% Similarity	Identity / Accession Numbers
1	Federal	Ita Oshin	1154	100	S.*enterica serovar typhi str* CT18 NC 003198.1
2	Federal	Aro	1160	85	S.*enterica serovar typhi str* TY2 NC 004631.1
3	Federal	Obada	1132	85	S.*enterica serovar typhi str* CT18 NC 003384.1
4	Federal	Obada	1094	70	S.*enterica serovar typhi str* TY2 NC 003385.1
5	Private	Akinolugbade	1093	71	S.*enterica serovar typhi str* CT18 U88545.1

6	Private	Olomore	1130	69	S.*enterica serovar typhi str* CT18 NC 021176.1
7	Private	Akinolugbade	1193	85	S.*enterica serovar typhi str* CT18 NC 016832.1

The percentage of titre values of Widal test positive patients selected for the study includes ≥ 1/80 (35.5%), ≥ 1/160 (52.0%) and ≥ 1/320 (12.5%) (Table 2). Out of the 200 stool samples collected, *Salmonella enterica serovar typhi* were isolated from 7(3.5%) of the samples. Of the 7(3.5%) of the total percentage of the *Salmonella enterica serovar typhi* isolated, 5(71.4%) were from patients age 0-18 while 2 (28.6%) were isolated from patients above 18 years. Also, 3(42.9%) isolates were from male and 4(57.1%) from female (Table 3). In terms of locality, occurrence rates were higher among people living in Ita Oshin 3(42.9%) and Obada 2 (28.6%) (Table 4). Also, there is higher occurrence among dependants 3 (42.3%) and students 2 (28.6%) (Table 5).

Comparing the results of different diagnostic techniques, out of the 200 true positive samples by Widal test method, only 7 (3.5%) were confirmed true positive by stool culture. Furthermore, all the 7 true positive samples by stool culture were further confirmed to be true positive samples by molecular method. Plate 1 shows the gel electrophoretic of 16S rRNA genes of isolated *Salmonella enterica serovar typhi* .

Table 2: Percentage of titre values of Widal test positive patients selected

Titre Value	**Number of Patients**
≥ 1/80	**71(35.5%)**
≥ 1/160	**104(52.0%)**
≥ 1/320	**25(12.5%)**
Total	**200(100%)**

Table 3: Distribution of *Salmonella enterica serovar typhi* isolated according to age and sex

Age	Male	Female	Number of Isolates
0-18	2(40.0%)	3(60.0%)	5(71.4%)
Above 18	1(50.0%)	1(50.0%)	2(28.6%)
Total	3(42.9%)	4(57.1%)	7(100.0%)

Table 4: Percentage of *Salmonella enterica serovar typhi* isolated according to Locality.

Locality	Number of samples	Number isolated
Obada	31(15.5 %)	2(28.6 %)
Ita Oshin	93(46.5 %)	3(42.9%)
Akinolugbade	67(33.5%)	1(14.3%)
Olomore	9(4.5%)	1(14.3 %)

Total	**200**	**7**

Table 5: Percentage of *Salmonella enterica serovar typhi* isolated according to occupation.

Occupation	Number of Samples	Number of isolates
Dependant	**78(39.0%)**	**3(42.3%)**
Student	**72(36.0%)**	**2(28.6%)**
Farmer	**5(2.5%)**	**1(14.3%)**
Civil Servant	**39(19.5%)**	**0(0.0%)**
Business	**6(3.0%)**	**1(14.3%)**
Total	**200**	**7**

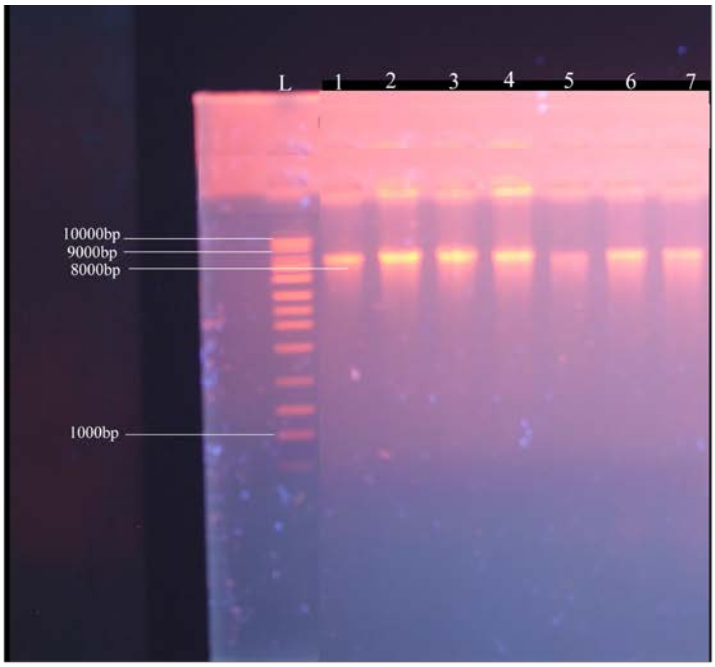

Plate 1: Gel Electrophoretic of 16S rRNA genes of *Salmonella enterica serovar typhi* Key :L=ladder,bp=base pair, 1-7:=Isolates 1-7

The phylogenic tree showed that six of the isolates formed separate clusters while one (1) of the isolate showed 100 % similarity with the isolates from NCBI database (Fig:1).

The isolates showed 100% resistant to azithromycin and ceftazidine, also 80% of the isolates were resistant to augumentin and gentamycin, while 50% and 40% of the isolates were resistant to ceftriazone and pefloxacin respectively (Fig: 2).

Furthermore, 90% of the isolates were sensitivity to ciprofloxacin, 80% to ofloxacin, 60% to both sparfloxacin and pefloxacin, 50% to cetriazone and 20% to gentamycin (Fig: 3).

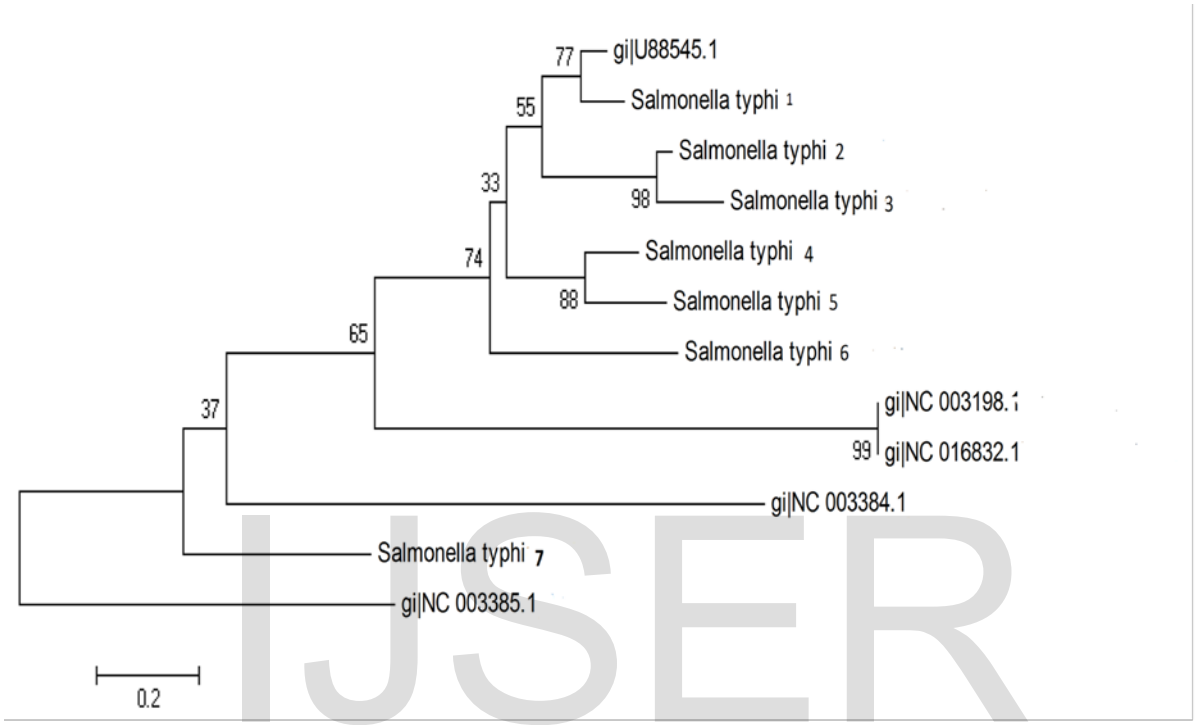

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Fig 1: Phylogenetic relationship among isolated *Salmonella typhi* based on 16S rRNA gene sequence from NCBI gene bank.

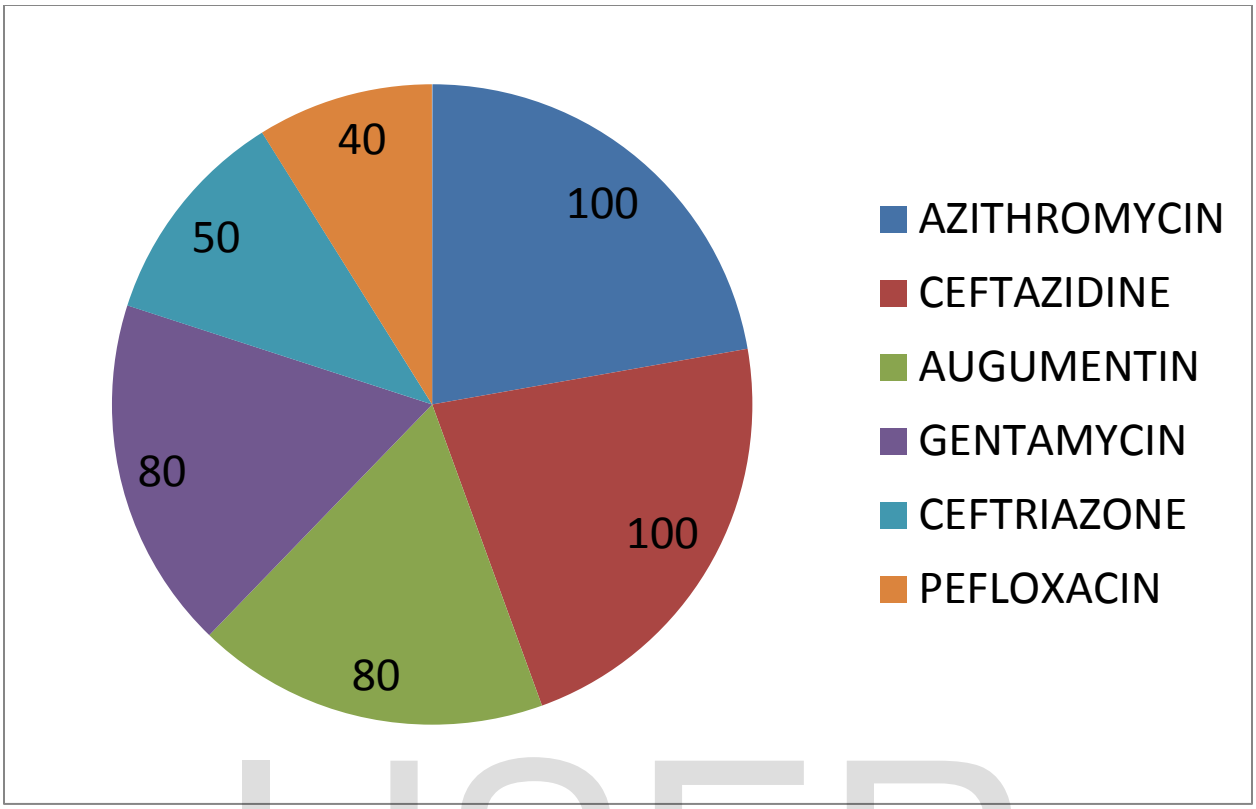

Fig 2: Percentage of resistant isolated *Salmonella enterica serovar typhi* to Antibiotics

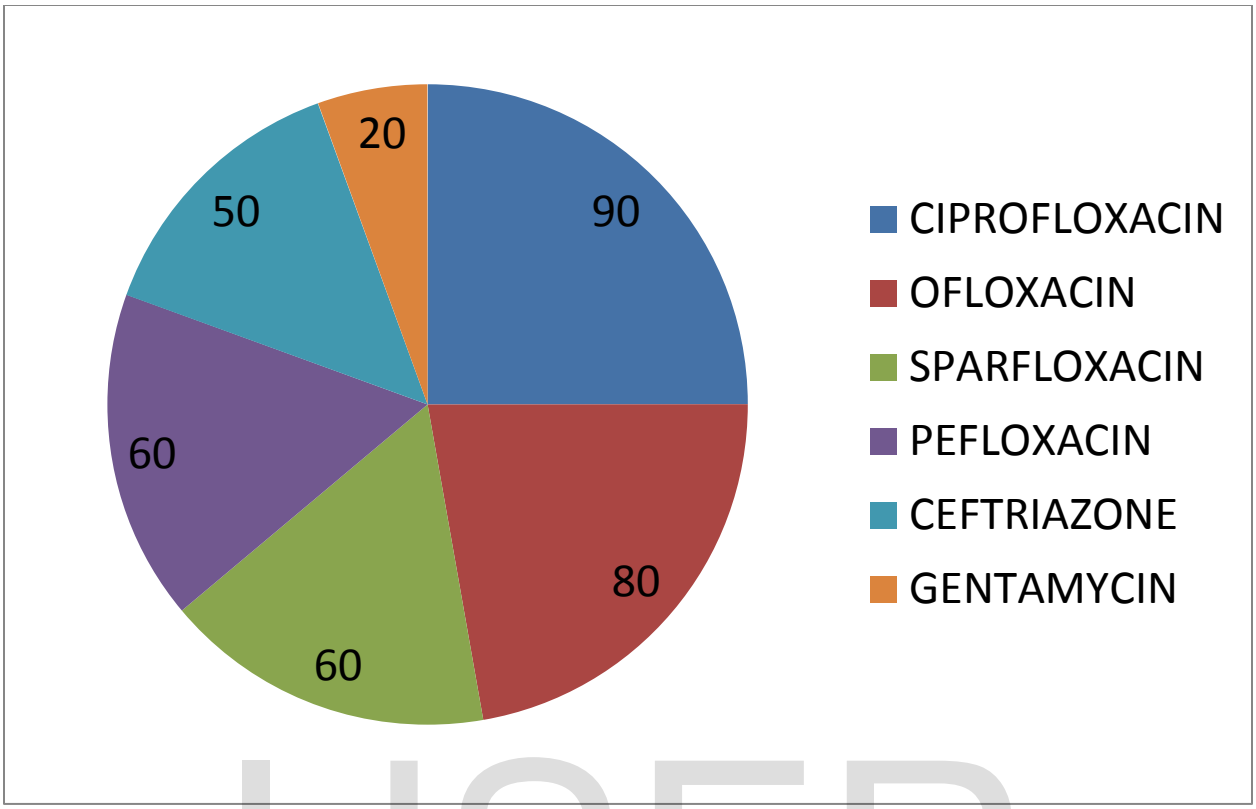

Fig 3: Percentage of Sensitive isolated *Salmonella enterica serovar typhi* to Antibiotics

5. DISCUSSION

The role of Widal test founded in 1896 by Georges Fernand Isidore Widal has been to increase the index of suspicions for the presence of typhoid fever by demonstrating a positive agglutination (Oluduro *et al.*, 2013). Unfortunately, in most developing countries including Nigeria the situation is quite different as the Widal test appears to be the only laboratory means employed in the diagnosis of typhoid fever among suspected patients. This test continues to suffer from serious cross-reactivity with other infectious agents that may produce false-positive results (Oluduro *et al.*, 2013). Since the ultimate goal of the test is antigen-antibody complex reaction, cross-reactions are encountered when antibody produced by nontyphoidal antigens reacts with typhoid-specific antigens (Ibekwe *et al.*, 2008).

This study reports molecular identifification of *Salmonella enterica serovar typhi* from stool culture of Widal test positive patients in selected Hospitals in Abeokuta using 16S rRNA gene amplification and sequencing techniques. A total of 200 stool samples were obtained from Widal test positive patients. All the patients had Widal test results of titre value $\geq$ 1:80.

All the isolates were confirmed and identified as *Salmonella enterica serovar typhi* based on cultural, morphological and biochemical tests. Further identification was done by molecular method using 16S rRNA gene amplification and sequencing. Seven (7) isolates of *Salmonella enterica serovar typhi* was cultured from the 200 stool samples given a prevalence of 3.5 %. This is in agreement with the observations of Adeleke *et al*. (2006) who reported low prevalence rate of *Salmonella typhi* in Zaria, Nigeria in a study on prevalence of typhoid fever and antibiotic susceptibility pattern of *Salmonella typhi.*

Akinyemi *et al*. (2000) also reported on evaluation of *Salmonella typhi* from blood collected from clinical diagnosed typhoid fever patients in the metropolis of Lagos, Nigeria that Widal testing done on acute phase serum of patients

suspected to have typhoid fever had limited diagnostic capability. This was because of its low sensitivity in which among all typhoid cases only 26% had diagnostic titre value. In addition, Eze *et al*. (2011) reported on prevalence of malaria and typhoid co-infections in Enugu State that malaria could interfere with serological diagnosis of typhoid and hence lead to over diagnosis of typhoid in Nigeria.

Out of the 7(3.5%) of the total percentage of the *Salmonella enterica serovar typhi* isolated, 5(71.4%) of the positive samples were isolated from patients age 0-18 while 2(28.6%) were isolated from patients above 18 years. Also, 2(28.6%) isolates were from male and 5(71.4%) from female. The high incidence reported from patients age 0 – 18 which are mostly children could be as a result of their food handling practice and hygiene because children could pick food directly from the floor (Zailani *et al.*, 2004). This result is in support of the study of Zailani *et al.* (2004) who reported highest incidence of antibody titre to *Salmonella typhi* in children in his work on effect of socio-economic status, age and sex on antibody titre profile to *Salmonella typhi* in Ile-Ife, Nigeria. This study is also in accordance with the work of Okonkwo *et al.* (2010) on the prevalence of *Salmonella typhi* among patients in Abeokuta, South- Western Nigeria who reported that more females had *Salmonella* agglutinin titres when compared with males.

Furthermore, this disagrees with the research conducted in Hong Kong by Kam *et al.* (2007) on molecular characterization of *Salmonella enterica serotype typhi*, he observed that males were more affected two times than the females. The main reason behind the probable discrepancies among different investigators may require more studies. Hence it could be explained or noted that *Salmonella* infection occurs irrespective of age or gender, appropriate precautions needed to be taken to completely prevent it (WHO, 2006).

In terms of locality and occupation, occurrence rates were higher among people living in Ita Oshin and Akinolugbade and among dependent and students. This study is in support of the work of Zailani *et al.* (2004) on the effect of socio-economic status, age and sex on antibody titre profile to *Salmonella typhi* in Ile-Ife, Nigeria who reported that typhoid and paratyphoid fevers are associated with occupation, poor environmental and living conditions especially in economically poor countries.

All the isolates were further identified as *Salmonella enterica serovar typhi* by molecular method using 16S rRNA gene amplification and seqencing. Molecular identification of the isolates using 16S rRNA gene amplication and sequencing showed that the seven (7) isolates from stool culture were *Salmonella enterica serovar typhi.* Molecular method of identification was reliable in the identification of *Salmonella enterica serovar typhi* from stool samples as all the isolates were identified as *Salmonella enterica serovar typhi.* In a related study, Achtman *et al.* (2012) showed that molecular methods had higher sensitivity and specificity of detecting *Salmonella species* when he isolated *Salmonella species* from fresh-frozen meat and poultry samples in Jordan using both molecular methods and conventional methods of identification.

The phylogenic analysis showed that six of the isolates formed separate clusters while one (1) of the isolate showed 100% similarity with the isolates from National Centre for Biotechnology Information (NCBI) database. Bacci *et al.*, (2014) suggests that virulence of *Salmonella* is linked to a combination of chromosomal and plasmid factors, which invariably may place them into separate clusters, therefore antimicrobial susceptibility tests before chemotherapeutic measure in the management of typhoid infection.

Comparing Widal test and stool culture in the diagnosis of typhoid fever, the stool culture remains the gold standard method because of its high level of sensitivity. The statistical analysis revealed significant differences ($p<0.05$) between the Widal test and stool culture in the diagnosis of typhoid fever. This however suggests that serological investigation (Widal test) alone is not reliable for the diagnosis of typhoid fever in humans. This report is also in support of that of Alfred and Comfort, 2004 on correlation studies on widal agglutination reaction and diagnosis of typhoid fever in Akwa Ibom, Nigeria who reported false positive results of Widal test in 51.28% of patients. Cunha, (2013) suggested that many of suspected cases of typhoid fever are actually chronic malaria infections, hepatitis or other immunological disorders.

The isolates showed 90% sensitivity to ciprofloxacin and 80% to ofloxacin and 60% to sparfloxacin and pefloxacin. However, all the isolates were resistant to azithromycin and ceftazidine while 80% of the isolates were resistant to both augumentin and gentamycin.

This study was in support of the observations of Adeshina *et al. (*2009) and Oluyege *et al*. (2013) who reported high sensitivity of ciprofloxacin and ofloxacin to *Salmonella enterica serovar typhi* while the level of resistant of *Salmonella enterica serovar typhi* to other commonly prescribed antibiotics is on the increase.

Furthermore, this study was contrary to the observations of Fashae *et al.* (2010) on antimicrobial susceptibility and serovars of *Salmonella* from chickens and blood samples of humans in Ibadan, Nigeria who reported multi drug resistance

of *Salmonella typhi* to three antibiotics (ciproflxacin, chloramphenicol and co-trimoxazole). Since the *Salmonella enterica serovar typhi* isolated in this study were from stool samples, whether or not sources of *Salmonella enterica serovar typhi* influences susceptibility to antibiotics was not clear and requires further investigation. However, Onuibgio in 1990 reported on misuse of the Widal test in the diagnosis of typhoid fever in Nigeria, he noted that the antibacterial resistance observed in the treatment of *Salmonella enterica serovar typhi* might be due to routine indiscriminate use of those antibacterial agents and/or rapid chromosomal mutation. In addition, the results interrelate with the findings of World Health Organization (WHO, 2006), that the treatment of *Salmonella* infection requires the use of more expensive Quinolones antibiotics such as oral ciprofloxacin. To successfully fight the increasing number of drug resistant and multi drug resistance bacteria, extensive knowledge of the molecular mechanisms of acquiring antibiotic resistance is required.

CONCLUSION AND RECOMMENDATIONS : This study demonstrated that stool culture and molecular identification of *Salmonella enteric serovar typhi* should be part of standard methods for the diagnosis of typhoid fever in humans. Stool culture should therefore be enforced as part of Good Laboratory Practice and evidence based diagnostic in the diagnosis and treatment of typhoid fever in humans.

Finally, while improved diagnostic tools have high priority in the management of typhoid fever, control measures should include provision of clean water and adequate community information to prevent infection, as well as offering vaccination to high-risk groups.

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