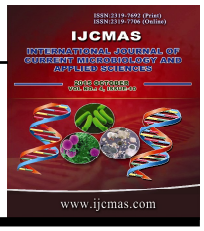
Int.J.Curr.Microbiol.App.Sci (2015) 4(10): 774-784

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 774-784**
http://www.ijcmas.com



Original Research Article

Isolation and Characterization of *Salmonella typhi* from Widal Positive Patients Attending Ekiti State University Teaching Hospital

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A B S T R A C T

The occurrence of typhoid fever in widal positive patients attending Ekiti State teaching Hospital between August to November, 2011 was investigated. Widal Agglutination test kit (serologic) was used to detect widal positive patient while brain heart infusion, Bismuth Sulphide agar were used to culture the blood samples from the patients. Demographic data were compiled from the patient's prescription card. The isolates were characterized using standard procedures. Out of the 99 samples examined, 86 were tested for Widal while the remaining 13 were cultured directly. Results of the Widal test showed that 42 (48.8%) had a titre of 1:160 and above. The disease was prevalent among the females, 18 (18.2%) females were positive as against 8 (8.1%) males. The disease had its highest frequency of occurrence within age group 20- 29 with 12 (13.59%) patients. Nine different bacteria genera were isolated from the blood samples. 13(36.1%) strains of *Klebsiella* sp. was found to be predominant. 7(19.4%) strains of *Proteus* sp, 4(11.1%) strains each of *Escherichia coli* and *Citrobacter* sp., 2(5.6%) strains each of *Shigella* sp., *Enterobacter* sp., *Salmonella paratyphi* B, and *Yersina* sp. while 1(2.8%) strain of *Providencia* sp. was isolated. Consequently, the disease has a relatively high occurrence rate in Ado-Ekiti from the Widal test result although the isolation rate of the aetiological agent was low.

Keywords

Salmonella typhi, Typhoid fever, Widal test, Serology

Introduction

Salmonella is a genus of rod-shaped Gram-negative non-lactose fermenting *Enterobacteriaceae* that cause typhoid fever, paratyphoid fever and food borne illness (Ryan and Ray, 2004). *Salmonella* serotypes are now placed under 2 species due to the difference in 16SrRNA sequence analysis, *Salmonella enterica* (2443 serotypes) and *Salmonella bongori* (20 serotypes). *S* subsp *enterica* is mainly isolated from warm blooded animals and account for more than 99% of clinical isolates whereas remaining subspecies and *S. bongori* are mainly isolated from cold-blooded animals and account for less than 1% of clinical isolates (Pui *et al*., 2011).

Typhoid fever is an endemic disease in the tropics and sub-tropics and has become a major public health problem in developing

countries of the world with an estimated annual incidences of 540 per 100,000,(Okonko *et al*., 2010). The annual incidence of typhoid is estimated to be about 17million cases worldwide (WHO, 2008). It is often encountered in tropical countries including Nigeria where they constitute serious sources of morbidities and mortalities (Ibekwe *et al*., 2008). Typhoid fever is among the major widely spread disease affecting the population in Nigeria and has been rated eight among these common infections (Anon, 1993). Nigeria, like many other tropical and developing countries has been described as endemic zone for typhoid fever by several researchers, (Obegbulan *et al*., 1995; Ikene and Anan, 1996).

Environmental and behavioural risk factors that are independently associated with typhoid fever include eating food from street vendors, living in the same household with someone who has new case of typhoid fever, washing the hands inadequately, sharing food from the same plates, drinking un-purified water and living in a household that does not have a toilet (Ram *et al.*, 2007; Ali *et al.,* 2006). The incidence of typhoid fever remains very high in impoverished areas and the emergence of multidrug resistance had made the situation worse (Marathe *et al.*, 2012).

Widal test a tentative diagnosis of typhoid fever is usually based on demonstrating the presence of agglutinins (antibody) in the serum of an infected patient, against H (flagella) and O (somatic) antigens of *S. typhi* (Olopoenia *et al.,* 2000). A 4-fold rise in O and H antibody titres in paired specimen obtained two weeks apart suggests *S. typhi* infection. Other means of diagnosing *S. typhi* includes culture of blood, urine, bone marrow and feaces.

Antibiotics such as Ampicillin, Chloramphenicol, Trimethoprim-sulfamethoxazole, Amoxicillin and Ciprofloxacin have been commonly used to treat typhoid fever in developed countries, where resistance is uncommon, the treatment of choice is fluoroquinolone such as ciprofloxacin (Parry and Beeching, 2009).Concomitant with the increase in diagnosis is the abuse of the first line drug of choice (Chloramphenicol) which has led to the selection of resistant strains of *S. typhi*. (Olopenia *et al*.,2000). Resistance to Ampicillin, Trimethoprim-sulfamethoxazole and Streptomycin is now common. Sanitation and hygiene are the critical measure that can be taken to prevent typhoid. Careful food preparation and washing of hands are crucial to preventing typhoid. In controlling typhoid, contact management and case management is essential. In generality, typhoid can be prevented through.

1. Ensuring access to safe water and appropriate human excreta disposal.
2. Emphasizing basic hygienic measures (i.e. proper hand washing after using the toilet, and before and after handling food) implementation and reinforcement.
3. Promotion of safe food handling practices like
   a. Thorough cooking of raw food from animal source
   b. Washing raw vegetables
   c. Washing hands, knives and cutting boards after handling uncooked foods.
4. Health education and community participation and public awareness.
5. Immunization: there are two vaccines currently recommendation by the World Health Organization for the prevention of typhoid fever (WHO, 2008). These are the live Ty21a vaccine for use in children aged 6 years and older and the

injectable typhoid capsular polysaccharide vaccine for use in children aged 2 years and older. Boosters are recommended every 5 years for the oral vaccine and every 2 years for the injectable form (WHO, 2008).

As Widal agglutination suffers from serious cross-reactivity with other infectious agents, it may produce false-positive results, leading to an over-estimation of the diagnosis of typhoid fever. This study is aimed at determining the occurrence of *Salmonella* sp. from Widal positive patients**.**

Materials and Methods

Study design, period and area

This was a cross-sectional sampling of Widal patients who attended the Ekiti State University Teaching Hospital and who had come for the Widal test between August and November, 2011. The inclusion criteria in the study were patients who had come for Widal test and patients recommended for blood culture. Those who had a titre of ≥1: 160 were considered as having active infection and were regarded as positive for Widal test.

Ethical consideration

Consent to collect samples and data from the Hospital was obtained from relevant authorities. The Ekiti State University Teaching Hospital Ethical Board gave permission to conduct the study within the hospital as stipulated by National Ethical Board.

Sample collection

A single blood culture either directly or from Widal patients was taken from all patients with suspected cases of typhoid referred to the Microbiological laboratory of Ekiti State University Teaching Hospital. A total of 99 blood samples were collected from patients who were suspected of having typhoid fever according to presumptive diagnosis by a medical practitioner. 2ml of venous blood were collected aseptically and inoculated onto 18ml of Brain Heart Infusion Broth Agar (Oxoid, England), which was incubated at 37^{o}C for 7 days (Stella *et al*., 2011).

Data management and analysis

Demographical data of patients with suspected cases of typhoid referred to the Microbiological laboratory of Ekiti State University Teaching Hospital were descriptively analysed using tables and statistical software (SAS). Analysis of Variance (ANOVA) was done and Duncan Multiple Test was used for the post hoc test at 95% confidence level.

Isolation

The bottles were examined daily for evidence of bacteria growth, including turbidity and haemolysis. The first sub-culture was done after 48 hours in Bismuth Sulfide Agar (Oxoid, England), sub-culturing was done on the same medium till the 7^{th} day. Brown and black colonies presumably *Salmonella* sp. was picked and characterized using standard methods.

Identification

Identification of *Salmonella* species was done biochemically using Kliger Iron Agar Agar (Oxoid, England) , Simmon Iron Medium Agar (Oxoid, England), Urea base agar (Lab M, England) and Simmons citrate agar (Lab M, England), were used to screen the isolates before serologic testing was performed (Cheesbrough, 2002).

Serological testing

Serologic identification of *Salmonella* species were performed using Wellcolex colour *Salmonella* test kit. One or two suspected *Salmonella* colonies from the culture plate were carefully emulsified in approximately 200µl of sterile saline in a suspension tube. Holding the bottle vertically, re-suspended latex reagent 1 and 2 were dropped into a separate circle on a flat reaction card after shaking vigorously for few seconds. About 40µl of bacterial suspension was transferred to two of the reaction circles containing latex reagent 1 and 2 respectively and mixed. The card was placed on a suitable flatbed rotator and run at 150 ± 5 rpm for 2 minutes then switch off and observed for agglutination without removing the card rotator. Positive controls with the positive control reagents (green, blue and red control) were carried out alongside with the latex reagent 1 and 2 respectively without the inoculums. Results were interpreted according to the manufacturer guidelines for usage of the kit.

Results and Discussion

Of the 99 blood samples collected in this study, the presumptive diagnosis of the patients showed that 86 were recommended for Widal and the remaining 13 for direct blood culture. The Widal test was used to detect four species of Salmonella namely *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C. For *Salmonella typhi*, only 3 (3.5%) had a titre of 1/320, 23 (26.7%) patients had a titre value of 1/160 and 14 (16.3%) had a titre of 1/80. Others (53.5%) had a titre value less than 1/40. For *Salmonella paratyphi* A, only 3 (3.5%) had a titre of 1/320, 3 (3.5%) patients had a titre value of 1/160 and 15 (17.4%) had a titre of 1/80.Others (75.6%) had a titre value less than 1/40. For *Salmonella paratyphi* B, only 3 (3.5%) had a titre of 1/320, 4 (4.7%) patients had a titre value of 1/160 and 11 (12.80%) had a titre of 1/80 while others (79%) had a titre less than 1/40. For *Salmonella paratyphi* C, only 1 (1.2%) had a titre of 1/320, 2 (2.3%) patients had a titre value of 1/160 and 9 (10.5%) had a titre of 1/80 while others (86%) had a titre less than 1/40. Overall, infections with *Salmonella typhi* were more prevalent with 23(26.7%) patients being infected with this particular species.

The result of the Widal positive patient is shown on Table 2. In this study, a total of 42 (48.8%) patients had a titre of 1:160 and above to the four species of *Salmonella* tested. Twenty-six (30.2%) of the patients examined were infected with *Salmonella typhi*, 7(8.1%) with *S. paratyphi* B, 6(7.0%) with *S. paratyphi* A and 3(3.5%) with *S. paratyphi* C. For *Salmonella typhi*, 3 (3.5%) had a titre of 1/320, 23 (26.7%) patients had a titre value of 1/160. For *Salmonella paratyphi* A, 1 (1.2%) had a titre of 1/320, 5 (5.81%) patients had a titre value of 1/16. For *Salmonella paratyphi* B, 2 (2.3%) had a titre of 1/320, 5 (5.8%) patients had a titre value of 1/160. For *Salmonella paratyphi* C, 1 (1.2%) had a titre of 1/320, 2 (2.33%) patients had a titre value of 1/160.The disease was found to be prevalent within the age group 20 - 29, with 12 positive patients, followed by adult age group with 6 positive and age group 30 - 39 having 2. Age group 10 – 19, 40 - 49, 50 – 59, 60 - 69 had 1 each while and none were positive in age group 0 - 9 and 70 – 79. The disease was found to be prevalent among the females with 18 (18.2%) of the patients examined as against 8 (8.1%) for males examined as shown in Table 3.

The Analysis of Variance (ANOVA) of positive patients examined across Age and Sex is shown in Table 4. The result showed there was significant difference in age distribution. The Duncan Multiple Test

further revealed that positive patients in Age bracket 20-29 having mean with alphabet A, was significantly different from the remaining age brackets (Table 5). While for the sex distribution there was no significant difference as both male and female positive patients' distribution had mean with same alphabet A (Table 6).

The profile of the organisms isolated is shown in Table 7. Of the 36 organisms isolated only 2 (5.6%) were confirmed to be *Salmonella* sp while; 13(36.1%) were identified to be *Klebsiella* spp.,13(36.1%) *Citrobacter* sp; 7 *Proteus* sp, 7(19.4%); *Escherichia coli* 4, (11.1%); *Yersinia* sp 1(2.8%); *Shigella* sp, 2(5.6%); *Enterobacter* sp, 2(5.6%); *Providencia* sp, 1(2.8%). In addition to the *Salmonella* sp., Going by the characterization and profile for the organisms isolated using the biochemical test results and biochemical reactions of some *Enterobacteriaceae* and other enteric organism (Cheesebrough, 2002),) was found to be dominant among the organisms isolated.

In this study, it was discovered that 42 (48.84%) patients were positive for Widal test (Table 2). This shows that the prevalence of the disease is relatively high in Ado-Ekiti which is in concord with the current view that typhoid fever is a disease of the developing countries (Ibekwe *et a*l., 2008) . This relatively high prevalent rate of the disease is due to the absence of municipal water supply, poor hygienic conditions, construction of toilets near source of drinking water, indiscriminate dropping of refuse and waste , lack of proper waste disposal system. Most residence in Ado-Ekiti lack access to portable water supply like pipe borne water and their source of drinking water is from the well.

During the course of this study, *Salmonella typhi* was found to be more prevalent among the *Salmonell*a strains recovered during Widal test since it was recovered from 26 (26.74%) of the 86 samples collected as compared to 6 (6.98%),7 (8.14%) and 3 (3.49%) obtained from *S. paratyphi* A ,*S. paratyphi* B and *S. paratyphi* C respectively (Table 2). This is in agreement with a similar finding by Sofia *et a*l., (2006). *S. typhi* has Vi antigen, a virulence factor that aids in its pathogenecity.

Furthermore, age group 20-29 and adult were observed to be highest among examined patients suspected for typhoid fever with 25 and 29 patients respectively out of the 86 patients examined for Widal. Also, it was discovered the same age groups had the highest prevalence rate among patients that were Widal positive. For the Widal positive patients examined, 12 were in age group 20-29, while 6 were adults. Futhermore, age group 20-29 were discovered to be significantly different statistically (Table 4 and 5). This is in agreement with a previous finding that typhoid fever in Nigeria is most common among the young adults and prevalent during the wet season. (Akinyemi *et al*., 2000). The prevalence may be because of their bad quality of drinking water, exposure to contaminated ready to eat food items available in open air cafeteria, reliance on fast food joints, lack of good hygienic practices, and common practice of consuming unwashed and partially cooked vegetables which possibly enhance the spread of the infection. Furthermore, it was discovered that of the Widal positive patients examined, none of them were from the age bracket 0-9 (Table 3). This may be an indication that typhoid fever in children under 5 years is low in the study area. Also, it was discovered that the infection was more prevalent among the females than the males in age group 20-29 and adult (table 3).

Of the 12 patients positive for Widal in age

bracket 20-29, 8 were females while 4 were males and the adult group with 6 Widal positive patients had 5 females and 1 male. *Salmonella typhi* was found to be more prevalent among the female than the males (table 3) with a prevalence rate of 18 (20.93%) as against 8 (9.30%) of the 86 samples examined, although there was no statistical significant difference among the gender distribution of the positive patients (Table 6). Again, of the 86 samples examined 17 were females out of 25 for age group 20-29 and 18 were females out of 29 for the adult age bracket. This is in contrast with similar findings by Sofia *et al.*, (2006). All these could be due to socio-cultural practices where best health care and food stuffs are preferentially provided to boys and men over girls and women and the high susceptibility of the female gender because of their anatomical structure and body physiology.

Table.1 Titre distribution of patients examined

	S. typhi	*S. paratyphi A*	*S. paratyphi* B	*S. paratyphi* C
1/20	44 (51.1%)	65 (75.6%)	67 (77.9%)	73 (84.9%)
1/40	2 (2.3%)	-	1 (1.2%)	1 (1.2%)
1/80	14(16.3%)	15 (17.4%)	11 (12.8%)	9 (10.5%)
1/160	23 (26.7%)	5 (5.8%)	4(4.7%)	2 (2.3%)
1/320	3 (3.5%)	1 (1.2%)	3(3.5%)	1(1.2%)
TOTAL	86 (100)	86 (100)	86 (100)	86 (100)

Table.2 Titre Distribution of Widal Positive Patients Examined Using a Titre Cut Off ≥ 1:160

	S. typhi	*S. paratyphi* A	*S. paratyphi* B	*S. paratyphi* C	Total
1/160	23(26.7)	5 (5.81)	5 (5.80)	2 (2.3)	35(40.7%)
1/320	3 (3.50)	1 (1.2)	2 (2.30)	1 (1.2%)	7(8.1%)
Total	26(30.23)	6 (6.98)	7(8.14)	3(3.49)	42(48.8%)

Table.3 Age and sex distribution of patients examined and those positive for Widal using a titre cut off ≥ 1:160 for *Salmonella typhi*

AGE GROUP	0-9		10-19		20-29		30-39		40-49		50-59		60-69		70-79		ADULT		TOTAL POSITIVE
	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	
Male	1	-	3	-	8	4	2	-	4	2	3	1	2	-	1	-	11	1	8
Female	-	-	5	1	17	8	5	2	3	1	1	-	1	1	-	-	18	5	18
Total	1	-	8	1	25	12	7	2	7	3	4	1	3	1	1	-	29	6	26

Legend: E: Number Examined, P- Positive Patients

Table.4 Analysis of Variance (ANOVA) of distribution of positive patients examined across age and sex

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Age	8	60.44444444	7.55555556	4.18	0.0294
Sex	1	5.55555556	5.55555556	3.08	0.1175

DF - Degree of freedom.
If PR F is less than 0.05, there is significant difference but if less than 0.05 there is no significant difference.

Table.5 Duncan multiple range test of distribution of positive patients examined across age

Duncan Grouping	Mean	N	Age
A	6.000	2	20-29
A			
B A	3.000	2	Adult
B			
B	1.500	2	40-49
B			
B	1.000	2	30-39
B			
B	0.500	2	50-59
B			
B	0.500	2	10-19
B			
B	0.500	2	60-69
B			
B	0.000	2	0-9
B			
B	0.000	2	70-79

Means with the same letter are not significantly different

Table.6 Duncan multiple range test of distribution of positive patients examined across sex

Duncan Grouping	Mean	N	Sex
A	2.0000	9	Female
A	0.8889	9	Male

Means with the same letter are not significantly different

Table.7 Profile of organisms isolated

Organism	Frequency	Percentage (%)
***Citrobacter* spp**	4	11.1
***Proteus* spp**	7	19.4
Escherichia coli	4	11.1
***Klebsiella* spp**	13	36.1
***Yersinia* sp**	1	2.8
***Shigella* spp**	2	5.6
***Enterobacter* spp**	2	5.6
***Providencia* sp**	1	2.8
***Salmonella* spp**	2	5.6
Total	36	100

Table.8 Serological test result

Isolate	Agglutination		Colour change		Result	
	Reagent 1	Reagent 2	Reagent 1	Reagent 2	Reagent 1	Reagent 2
1	+	+	Red	Red	*S. paratyphi B*	Vi antigen
2	+	+	Red	Red	*S. paratyphi B*	Vi antigen

The importance of using blood culture in the isolation of the aetiological agent of typhoid fever as definitive diagnosis cannot be overemphasized. Although, Widal test had been used as a presumptive test to increase the suspicion index for typhoid fever, most suspected cases of typhoid fever have been erroneously confirmed using the antibody titre based on single serological test. Other infectious agents such as bacterial, viral and even protozoan may mimic enteric fever (Christie, 1974, Akinyemi *et al*., 2002, 2004). Disease such as malaria, non-typhoidal Salmonellosis, Endocarditis, Parasitemia and other gastro-intestinal infections may have been responsible for the high antibody titre since studies carried out by Akinyemi *et al* (2004) indicates that malaria parasitemia also mimic and interfere with the "O" and "H" antigens of commercial available Widal agglutination kits.

From this study, 9 genera of bacteria were isolated and characterized based on the biochemical characteristics of Sugar utilization, Indole production, Citrate and Urease utilization, Sulphide and Gas production. Of the 36 organisms isolated only 2 (5.6%) were confirmed to be *Salmonella* sp while; 13(36.1%) were identified to be *Klebsiella* spp.,13(36.1%) *Citrobacter* sp; 7 *Proteus* sp, 7(19.4%); *Escherichia coli* 4, (11.1%); *Yersinia* sp 1(2.8%); *Shigella* sp, 2(5.6%); *Enterobacter* sp, 2(5.6%); *Providencia* sp, 1(2.8%).(table 7 and 8). Only 2 isolates were *Salmonella Paratyphi* B of the 45 isolates recovered from the blood culture (Table 8). This, nearly negative culture results could be because of the inability to ascertain the onset of the disease since culturing could have being done at the early stage of the infection or after the onset, when the organism has caused bacteremia in blood and have

migrated to the gastrointestinal tract. More so, most of the patients might have taken antibiotics before seeking medical care at any health centre. This indiscriminate use of antibiotics due to rampant practice of self-prescription among patients might have inhibited the growth of susceptible bacteria. Gross abuse of antibiotics among Nigerians had been well documented by several workers (Olukoya *et a*l., 2000; Wallinga, 2002; Grossan, 2003). More so, only 2ml of blood was collected from the patients examined. The use of antibiotics before consultation and the low volume of blood samples collected could have caused the relatively low culture rate (Mochammed *et al*, 2007).

Isolation of other organisms suggested that most of the patients will not respond to treatment if placed on these first line drugs. It indicates that some of the patients may not respond to treatment if they are placed on a regime strictly for typhoid fever. Wide variation in sensitivity pattern of various strains makes it necessary to assess the sensitivity of Typhoid Bacilli to antibiotics before instituting therapy. Incomplete treatment may also be factor contributing to development of resistance.

Blood culture is most sensitive during the early stage of infection when the pathogen is still circulating in the vascular system. The sensitivity of blood culture decreases with the duration of the illness (Stella *et al*., 2011). The isolation of *Salmonella typhi* from blood culture is significantly higher in patients whose blood sample was taken after 4 days of the fever (Feng-Ying *et al*., 2000).

In this study, the prevalence of typhoid fever was found to be relatively high in Ado-Ekiti from the Widal result, though the isolation rate of the aetiological agent was low. Furthermore, the blood is sterile. The isolation of other organisms in the blood culture from this study is an indicator that the symptoms observed in these typhoid suspected patients were caused by these isolated organisms. Wide variation in sensitivity pattern of various strains makes it necessary to assess the sensitivity of Typhoid Bacilli to antibiotics before instituting therapy. Incomplete treatment may also be factor contributing to development of resistance.

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