(RESEARCH ARTICLE)

Cultural, serotyping and plasmid profile of Salmonellae in Lagos, Nigeria

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Abstract

Problems associated with typhoid fever epidemic about its diagnosis in developing countries like Nigeria is a perennial healthcare challenge the healthcare sector grapples with. Improper diagnosis of clinical cases have also led to treatment failure and errors as diseases caused by other microorganisms are treated as typhoid fever especially as a result of inadequate reliable diagnostic laboratories. A total of 3,000 stool specimens from patients were analyzed using standard microbiological techniques. Of this, 1,391 *Salmonella* spp. were recovered, constituting 233 (88.14%) *S. typhi* while 158 (11.36%) were non-typhoidal *Salmonella. S. typhi* was recovered from more females, 685(55.6%), than males, 548 (44.4%). The 41 and above age group had the highest incidence of *S. typhi* of 220(17.8%) in females as against 280 (22.7%) in males within the 21-30 age group. Antibiotic susceptibility testing using the disc diffusion method by Kirby Bauer showed high multiple resistance to most of the 15 different antibiotics tested but susceptible to the first line typhoid fever drugs (chloramphenicol 85%, cotrimoxazole 86.7%, ampicillin 88.3% and amoxicillin 90%) and highly susceptible to third generation cephalosporins and fourth generation fluoroquinolones. The *S. typhi* tested showed four different resistance patterns. Plasmid profile analysis of 200 multiple antibiotic resistant *Salmonella* isolates identified culturally and biochemically as *S. typhi* but by serotyping showed *Salmonella* other than *S. typhi* were erroneously classified as *S. typhi*. Majority of the *S. typhi* harbored mostly small sized plasmids which ranged from 2.2 Kb to 55.5 Kb. It can be deduced from this study that multiple drug resistance in *S. typhi* is likely to be plasmid mediated. The eleven antibiotic resistance patterns were reduced to eight plasmid clones indicating the diagnostic efficacy of plasmid profiling over the former method.

Keywords: Cultural; Morphological; Biochemical; Salmonella; Serotyping; Plasmid profile

1. Introduction

Diagnosis of typhoid fever is carried out by several methods in order to identify the causative agent- *Salmonella typhi* among which is serotyping. Other methods include blood cultures, stool cultures, urine cultures and molecular methods including polymerase chain reaction (PCR). Definitive diagnosis of salmonellosis depends on isolating the etiologic agents from clinical samples (specimens) which include stool, blood, urine and bone marrow [1]

Salmonellae can as well be isolated from the cerebrospinal fluid (CSF) in case of *Salmonella* meningitis aspirates from the chest and bone marrow in *Salmonella* chest infection and cases of osteomyelitis [2]. Blood cultures in untreated patients are most likely to be positive during the period of established disease or in the period preceding late stage of active invasion by the organism between7-10 days of acute illness and up to the second week [3]. Blood is either inoculated directly into selective media or onto enrichment media like brain heart infusion (BHI) broth, buffer peptone water (BPW), selenite f broth, Columbia broth and trypticase soy broth before inoculation into selective media, for optimal results [4].

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Stool samples are best obtained during the acute diarrheal phase when *Salmonella* is present in the stool in appreciable number, usually the second through the fourth week of illness. The advantages of pre-enrichment of stool with special media such as Rapparport-Vassiliadis medium and enrichment using SF broth and/or tetrathionate broth prior to culture on solid selective media over direct inoculation investigation of outbreaks or carrier is noteworthy. Its promotion of the growth of injured cells of *Salmonella* have been reported by several authors [4, 5, 6]. Serological techniques are used to identify unknown cultures with known sera and may also be used to determine antibody titers in patients with unknown illness. These are usually agglutination tests and are classified into two: 1) Rapid slide agglutination used for rapid preliminary identification of cultures and 2) Tube dilution agglutination test otherwise called Widal test [4, 7].

The Widal serological method has become most frequently and widely used thus called the 'popular' method of diagnosing typhoid fever in Nigeria. However, paired sera for the demonstration of 4-fold rise in antibody level which is required are hardly collected in Nigeria [8]. Rather, laboratory confirmation is usually based on the result of a single Widal test on serum samples collected during acute phase of the illness. The reliability of such single serum test depends on a proper interpretation of the result based on foreknowledge of the previous vaccination status and distribution of baseline levels of various typhoid agglutinins in the community and locality where the patients resides [9].

Widal test detects antibody to 'O', 'H' and 'Vi' antigens and suffers from low specificity, as majority of healthy population in developing countries possess antibodies. Of the three antibodies, only the 'O' and 'H' antibodies are produced during *S. typhi* infection and are measured by Widal agglutination reaction. Detection of antibody to lipopolysaccharide (LPS) and other proteins by the enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), counter-immunoelectrophoresis, indirect-immunofluorescence and slide test are reliable but not readily available [10].

Plasmids are autonomously self-replicating extra-chromosomal DNA elements, which are not essential for normal bacterial growth but can carry antibiotic resistance or toxin genes [11].The approach to using plasmid profiles as an epidemiological typing system is similar to that of other typing schemes. The ideal situation would be to show that all of the outbreak-associated strains have same plasmid profile and are the same by any other typing scheme used [12] .

One advantage of plasmid profiles over many other typing systems is that a set of reagents and equipment is applicable to many species of bacteria unlike phage typing which requires a battery of phage indicator strains and standardization or serotyping which requires a battery of antisera difficult and very expensive to acquire, prepare and standardize [13].

Antimicrobial susceptibility testing has the advantage of being done in a large number of laboratories and being highly standardized. The susceptibility pattern can be related to the plasmid content because of the wide variety of resistance genes that are plasmid encoded. Plasmid profiles often offer a higher level of sensitivity in distinguishing between antibiotic resistance patterns [11]. Just as antibiotic resistance pattern can be related to the organism's plasmid content, the serotypes, phage susceptibility or other phenotypic characteristics or metabolic properties may be plasmid encoded. However, plasmid profiles are most useful when combined with other methods of typing or screening, hence this study. This study carried out isolation, characterization, investigation, antibiotic susceptibility pattern and plasmid profiles of *Salmonella* isolates from patients in Lagos, Nigeria.

2. Material and methods

2.1. Bacteriological analysis

During a 2 year period from January 2017 to December 2019, a total of 1,233 *Salmonella* isolates which were recovered from stool samples of patients collected aseptically from patients and cultured on selective media which included MacConkey (MA) agar, xylose lysine deoxycholate (XLD) agar, Salmonella-Shigella (SS) agar (Difco, USA) and blue green deoxycholate agar (BGDA). Five grams of each stool sample was mixed with 45 ml of BPW and incubated for 24 h at 37^0C. After incubation, 0.1 ml of sample was inoculated into 10ml of Rappaport Vassiliadis R10 broth (RV, Merck, Germany) and then incubated for 24 h at 42^0C. One loopful of RV culture, which changed in color from blue to discolored or green, was streaked onto the surface of MA, XLD, SSA and BGDA (Difco, USA) plates, and the suspected colonies were identified to be Salmonella by means of biochemical tests and cultured on NA slopes and kept at 4^0C till use.

2.2. Antibiotic susceptibility testing

In the present study, antibiotic susceptibility testing was done on Mueller Hinton agar using disk diffusion (Kirby Bauer's) method according to the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines using the following antimicrobial drugs which concentrations were in micrograms: Amoxycillin (10), ampicillin(10), colistin

sulphate (25), chloramphenicol (10), gentamycin(10), nitrofurantoin (20), cotrimoxazole (25), nalidixic acid (30), streptomycin (25), tetracycline (25), kanamycin (30), cefotaxime (30), cefuroxime (30), ofloxacin (10), perflacin (10) and phenolprofloxacin (10).

2.3. Serotyping

Salmonella isolates were further subjected to immunological test using *Salmonella* antisera (Denka Seiken, Japan) to detect their somatic (O) and flagella (H) antigens. This allowed the determination of the species of the organisms using the Kauffman-White classification table [15]. Further phenotypic identification was performed using the VITEK GNI+ (bioMerieux, France) and API 20E identification system [13].

2.4. Plasmid profile and curing

Plasmid DNA profile and curing assay for antibiotic resistant bacterial isolates were carried out as described by Birnboin and Doly [16, 17, 18] and Zhou *et al*[18] with some modifications. To isolate the plasmids, a volume of nutrient broth was inoculated with aliquot collected from overnight bacterial culture grown in nutrient broth containing antibiotic for 24 h at 37^0C. The inoculated nutrient broth was incubated for 18-24 h to allow the growth of the bacteria. One and a half milliliter (1.5 ml) of an overnight culture of *S. typhi* was spinned in a micro-centrifuge (Microfuge, Biofuge A, Haraeus Sapatech, Germany) to pellet cells for 10 seconds at 7000 rpm. The supernatant was gently decanted leaving 50-100µl of supernatant. About 3000µl of TENS was mixed for 2-5 seconds until when the mixture becomes sticky. If more than 10 minutes were needed before moving to the next step, samples were kept on ice to prevent degradation of chromosomal DNA which may be precipitated with plasmid DNA.

One hundred and fifty microliter (150µl) of 3.0M sodium acetate with pH 5.2 were added and vortexed for 2-5 seconds to mix completely. The mixture was spinned in a micro-centrfuge to pellet debris and chromosomal DNA. The supernatant was transferred to a fresh tube, mixed well with 0.9 ml of 100% ethanol which has been re-cooled to -20^0C and was spinned for 2 minutes to pellet plasmid DNA and RNA (white pellet) were observed. The supernatant was discarded and the pellet rinsed twice with 1 ml of 70% ethanol. The pellet was dried under vacuum for 2-3 minutes and was then suspended in 20-40 ml of Tris-EDTA (TE) buffer and distilled further [17]. Agarose gel was run and plasmid DNA visualized on UV trans-illuminator. Molecular weight of plasmids were determined by comparing them with plasmid standard which sizes ranged between 2.2 to 7.5 Kb.

In curing plasmid DNA, a volume of nutrient broth was inoculated with aliquot collected from an overnight bacterial culture grown in nutrient broth containing antibiotics for 24 h at 37^0C. The inoculated nutrient broth was incubated for 18-24 h for bacterial growth. Sodium dodecyl sulphate (SDS) curing agent was added to bring the concentration to 1% (w/v) SDS followed by incubation of 24-48 h at 37^0C ensuring there was growth. Freshly prepared nutrient broth was inoculated with an aliquot of cured culture and incubated for 24 h at 37^0C. Plasmid DNA was isolated from the broth culture as described above. Agarose gel electrophoresis was run and plasmid DNA visualized on UV trans-illuminator to confirm curing.

3. Results

Bacteriological analysis of 3,000 stool samples was carried out using standard microbiological techniques. Majority of the patients, 11-20(20.27%), 40 years and above (12.00%), 21-30 (9.33%) and 31-40 (8.33%) age groups (Table 1).

Table 1 Age and sex distribution of subjects. Patients (n=3000)

Age	Male (%)	Female (%)
0-10	72(2.4)	96(3.20)
11-20	608(20.27)	375(12.5)
21-30	280(9.33)	215(7.71)
31-40	250(8.33)	168(5.60)
41 & above	360(12.00)	576(19.20)
Total	1570(52.33)	1430(47.67)

The male constituted 52.33% as against 47.67% females. A total of 1,391 *Salmonella* spp. were isolated, 1,233 (88.64%) were *S. typhi* while non-typhoidal Salmonellae (NTS) were 158 (11.36%). *S. typhi* was isolated from 685 (55.56%) females as against 548 (44.40%) males, 21-30 age group had 280 (22.80%) isolates among males while 41 and above age group had the highest incidence of 220 (17.84%) in females (Table 2).

One hundred and fourty two (61.74%) of the 115 *S. typhi,* 32(13.79%) were *S. paratyphi* A, 30 (13.04%) were *S.paratyphi* B and 12 (5.22%) *S. typhimurium (*Table 3).

Table 2 Occurrence of Salmonellae among subjects. Patients n=3000

Age	Male (%)	Female (%)	Total (%)
0-10	22(17.8)	40(3.24)	64(5.19)
11-20	100(8.11))	80(6.49)	180(14.59)
21-30	280(22.7)	200(16.22)	480(38.93)
31-40	80(6.49)	145(11.75)	225(18.25)
41 & above	64(5.19)	220(17.84)	284(23.03)
Total	548(44.4)	685(55.56)	1233(100)

Table 3 Incidence of Salmonellae (230) isolates using serotyping.

Species	Number	Percentage
S. typhi	142	61.74
S. paratyphi B	30	13.04
S. paratyphi C	14	6.09
S. paratyphi A	32	13.91
S. typhimirium	12	5.22
Total	230	100

Table 4 shows that the *S. typhi* tested showed high multiple drug resistance to tetracycline, colistin sulphate and kanamycin while they showed 100% susceptibility to the third generation cephalosporins and fourth generation fluoroquinolones.

The range of the various sizes of the plasmids which ranged from 2.2 Kb to 55.5 Kb is shown in Figure 1.
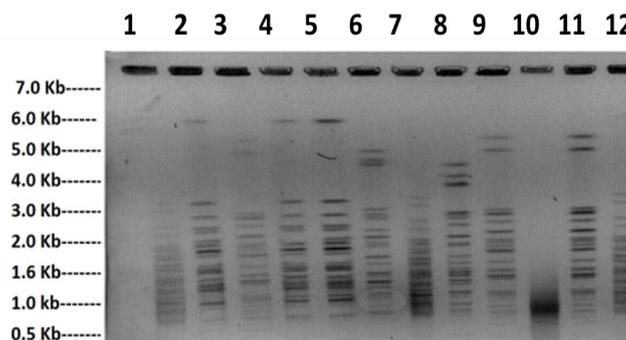
Figure 1 Agarose gel electrophoresis of plasmid DNA of *S. typhi* from patients

Lanes 1, 3 to 10 are plasmid DNA of *S. typhi* isolated from patients.
Lane 2 is the control *E. coli* V157

Table 4 Antibiotic susceptibility pattern of *S. typhi* (N=60).

Name of antibiotic	Susceptible	Resistant
Ampicillin	53(88.33)	7(11.67)
Chloramphenicol	51(85)	9(115)
Colistin sulphate	1(1.67)	59(98.33)
Gentamycin	60(100)	0(0)
Streptomycin	45(75)	15(25)
Nitrofurantoin	28(46)	32(53.33)
Cotrimoxazole	52(86.67)	8(13.33)
Tetracycline	0(0)	60(100)
Kanamycin	2(3.33)	58(96.67)
Augmentin	52(86.67)	8(13.33)
Amoxycillin	54(90)	6(10)
Cefotaxime	60(100)	0(0)
Peflacin	60(100)	0(0)
Ceftriaxone	60(0)	0(0)
Ofloxacin	60(100)	0(0)

Plasmid profile patterns of *S typhi* recovered showed the presence of many plasmids. Majority of the isolates studied harbored plasmids most of which are of low molecular weight. Of the 120 *S. typhi* isolates analyzed, 98 (90%) harbored 2.2 Kb plasmids, 32 (26.7%) harbored 2.8 Kb and 3.0 Kb plasmids respectively, while only 8 (6.7%) harbored 4.0 Kb and 5.2 Kb respectively (Table 5).

Table 5 Plasmid profile of Salmonella typhi from subjects. (Patients n=71)

Plasmid	Frequency (Percentage)
2.2 Kb	64(90.1)
2.8 Kb	19(26.8)
3.0 Kb	19(26.7)
4.0 Kb	5(7.0)
5.2 Kb	12(16.9)
5.5 Kb	24(33.8)
55.5 Kb	21(29.6)

4. Discussion

Salmonella infections have been reported to occur sporadically in different parts of Nigeria, the source of which have been very difficult to trace [19]. This study shows that *S. typhi* can easily be recovered from stool samples of patients using the pre-enrichment and enrichment techniques. This finding agrees with report by Oboegbulam [20] that *S. typhi* is easily recovered from stool probably by direct inoculation owing to overgrowth by other bacterial flora in the stool. The incidence of *S. typhi* (41.1%) was more prevalent in Lagos than other *Salmonella* spp. put together, a finding which corroborates report by Onile and Odugbemi [21] that *S. typhi* constituted 51.7% of their *Salmonella* isolates. *Salmonella typhi* infection would even be speculated to be probably more fulminant in Lagos than other areas in Nigeria where the

other *Salmonella* spp. with milder infections are prevalent. This finding is at variance with report by Niemogha [22] which discrepancy may be due to timing of the studies, methods, media used and the type of subjects used for each study.

Nigerians are predisposed to typhoid fever as a result of low technological and biological advancement, poor personal hygiene and medical facilities. *S. typhi* was more prevalent in the women within the 21-30, 31-40, and above 41 age which constitute 55.56% as against 0-10 and 21-30 age groups totaling 44.44%.

The *Salmonella* serotypes isolated from this study in Lagos was compared with those reported in other parts of Africa. These findings show that *Salmonella* serotypes in Lagos were mainly *S. typhi* 71(61.74%) as against Dakar, Senegal (55.%), Yaounde, Cameroon (70%), with *S typhimurium* having 85% of all the *Salmonella* in Libreville, Gabon, while in Ibadan, Nigeria, *S. typhi* was the most common serotype in humans [20].

Cases of human infections with *Salmonella* have been reported throughout the world but most previous reports on salmonellosis in Nigeria have concentrated on the clinical presentation and immunological tests as basis for diagnosis particularly using Widal test for the purpose of chemotherapy without evidence [21]. Contrary to report by Oboegbulam [*21*], a higher incidence of *S. paratyphi* B than S. *paratyphi* C was observed in this study. The occurrence of *S. paratyphi* A agrees with the situation in other countries like Britain and U.S.A. where *S. paratyphi* A is the most common serotype while *S. paratyphi* B and *S. paratyphi* C are rarely encountered and that infection with *S. typhi* is usually acquired overseas [24].

It was observed from this study that *S. typhi* with identical antibiotic resistance patterns differed markedly in their plasmid content, which is consistent with other reports [24, 25]. *S. typhi* isolates studied showed eleven resistance patterns and eight plasmid profiles among the isolates from patients. This finding agrees with an earlier report of high occurrence of plasmids in most of their isolates [26]. This observation suggests that plasmids are likely to play a prominent role in the pathogenicity of the *S. typhi* recovered in this study. Although molecular methods are adequately distinctive in classifying Salmonellae serotypes into sub-species in epidemiological studies, very few molecular studies had been conducted in Turkey, which is applicable to Nigeria [27].

More antibiotic susceptibility patterns as against plasmid profiles were encountered among the *S. typhi,* which is an indication that a large number of *Salmonella* clones are present in the environment [24].

Among the molecular based techniques used, plasmid profile analysis, random amplified polymorphic DNA analysis (RAPD), repetitive extragenic polymorphic sequences analysis by PCR (repPCR), and pulsed field gel electrophoresis (PFGE) are commonly used [28]. Plasmid survey of *Salmonella* strains has been used as an epidemiological tool in the investigation of outbreaks of enteric diseases, differentiation of strains or identification of sources of infection [25]. It has also been used as a fingerprint in discriminating between strains with identical antibiotic resistance pattern. There is however, paucity of information on the antimicrobial susceptibility pattern of bacteria in relation to their plasmid contents [12, 26].

Most of the *S. typhi* that harbored small molecular weight plasmids showed resistance to only a few antibiotics while those with larger molecular weight plasmids exhibited a high level of multiple resistance. This findings corroborate earlier report that plasmid profiles offer a higher degree of sensitivity in distinguishing between antibiotic resistance patterns [13]. It is thus very likely to deduce from the study that multiple antibiotic resistance among most of the *Salmonella* isolates were plasmid mediated. Plasmid analysis and pulse field gel electrophoresis were successfully used to isolate and investigate Salmonella serotype Enteritidis [29]

Multidrug resistance by bacteria has been ascribed to other factors such as integrons, which are a group of apparently mobile elements that can contain one or more antimicrobial resistance genes, and have been found in a variety of bacteria are thought to be largely responsible for the characteristic increase in multidrug resistant bacteria [30]. Detection of multidrug resistant *Salmonella enteritidis* which possessed plasmids containing class I integrons that code for the synthesis of extended spectrum B-lactamases had been reported [31].

5. Conclusion

This study shows the high incidence rates of the etiology of typhoid fever (*S. typhi*) thus confirms its endemicity in the study area. The isolates were highly susceptible to most of the first line drugs for the treatment of diseases caused by *S. typhi* including chloramphenicol, ampicillin, amoxicillin and cotrimoxazole but were highly resistant to other antibiotic tested. More resistance patterns but fewer plasmid profiles were found thus indicating that plasmid profile analysis

appear to be more discriminatory and of higher diagnostic efficacy. *S. typhi* harbored mostly plasmids with the lowest molecular size as well as those with higher molecular DNAs, thus suggestive that plasmid may play a role in the pathogenesis and virulence of the *S. typhi* from patients.

Compliance with ethical standards

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