Antibiotic Resistance of *Salmonella* Typhi from Stool of Patients Attending Selected General Hospitals in Nasarawa West Senatorial District, Nasarawa State

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ABSTRACT

The severity of *Salmonella* infections in humans varies depending on the serotype involved and the health status of the human host. This study was aimed at Molecular characterization of azithromycin resistant *Salmonella typhi* isolated from patients attending selected General Hospital in Nasarawa State. Stool samples were collected from patients attending selected General Hospital in Nasarawa State and Standard microbiological method were used in isolation and identification of *Salmonella typhi*.The antibiotic susceptibility test for the isolates was carried as described by clinical and laboratory standard institute. The overall occurrence of *Salmonella typhi* among patients attending selected General Hospital was 38(12.0%). The highest occurrence of *Salmonella typhi* was general hospital (19.3%) but lowest in Keffi general hospital (6.5%). The occurrence of the isolates in relation to the gender of patient's was higher in female (13.4%) than in male (10.1 %). The occurrence of the isolates in relation to age was high in 21-30 yrs (21.4%) but low in 41-50 yrs (7.6 %). The isolates were highly resistant to tetracycline (81.5 %) but less resistant to cefurazime (28.9%), ciprofloxacin (15.7%) azitharmicine (10.5%) and gentamicin (13.1%). The commonest resistance profile in the isolates were; CTX-CAZ-AMC-TE-CTX-CAZ-S-SXT-AMC-NA-C-TE and CTX-CAZ-S-SXT-AMC-NA-CN-CIP-C-TE with the percentage occurrence of 12.8%. The commonest MAR index in the isolates was 0.8 (33.3%).

Keywords: *Salmonella* infections, patients, antibiotic, resistance, female and male

INTRODUCTION

The *Salmonella* genus comprises two species, *S. enterica* and *S. bongori*, and more than 2,600 serovars [1], which can be grouped into typhoidal and nontyphoidal *Salmonella* (NTS) serovars [2]. The typhoidal *Salmonella* serovar, *S.* Typhi, and paratyphoidal serovars, *S.* Paratyphi A, *S.* Paratyphi B, *S.* Paratyphi C, and *S.* Sendai, can cause invasive systemic infections in humans and higher primates, resulting in an estimated 21,650,974 cases of typhoid fever and 216,510 deaths, along with 5,412,744 cases of paratyphoid fever, globally in 2019 [3]. NTS is estimated to cause 93.8 million illnesses, of which 80.3 million are foodborne, and 155,000 deaths each year [4]. Invasive NTS disease is a major cause of global morbidity and mortality, with the highest incidence in sub-Saharan Africa [5]. NTS is estimated to cause 535,000 cases of invasive disease, with 77,500 deaths globally in 2017 [5]. Among foodborne pathogens, NTS is the second leading cause of illness and the largest cause of hospitalization and death in the United States [6].

Nontyphoidal *Salmonella* infections in healthy humans usually result in only a mild and self-limiting symptomatic illness. Antimicrobial therapy can prolong the duration of excretion of NTS, and there is no evidence of benefit for antimicrobials in NTS diarrhea in healthy people; therefore, antimicrobial therapy is only recommended for people with severe illness, invasive disease, or certain risk groups, including infants, the elderly, and immunocompromised individuals [7]. Ampicillin, chloramphenicol, and cotrimoxazole used to be the first-line antimicrobials to treat salmonellosis [8]. Due to the widespread resistance of Salmonella serovars to conventional first-line drugs, fluoroquinolones (e.g., ciprofloxacin), third-generation

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cephalosporins (e.g., ceftriaxone), macrolides (e.g., azithromycin), and carbapenems (e.g., meropenem) have been indicated as the critically important antimicrobials for the treatment of salmonellosis [8,9]. Thus, surveillance of the resistance to the critically important drugs in *Salmonella* isolates is of great medical concern. In the United States, resistance in human typhoidal *Salmonella* serovar isolates from 2014 was low to ceftriaxone (2.4%) and very rare to ciprofloxacin (0.4%) and azithromycin (<0.1%) [10]. In Europe, resistance in human typhoidal *Salmonella* serovar isolates from 2020 was 14.1% for ciprofloxacin, 0.8% for cefotaxime and ceftazidime, and 0.8% for azithromycin [11].

Azithromycin, a semisynthetic macrolide, has been widely used to treat a variety of bacterial infections, including invasive salmonellosis [12]. This broad-spectrum agent had been used in massive treatments to eradicate trachoma and reduce all-cause mortality in children [13]. However, massive use of azithromycin selects for resistance to this antimicrobial in bacteria [14]. Although azithromycin resistance in typhoidal *Salmonella* serovar is rare, resistance has been increasing over time and seems to be more prevalent in strains with multidrug resistance and fluoroquinolone resistance [15].

Macrolides, such as azithromycin and erythromycin, inhibit bacteria by binding to bacterial 50S ribosomal subunits to hinder mRNA translation [12]. Bacteria can develop resistance to macrolides, including azithromycin, through target alterations in 23S rRNA and ribosomal proteins L4 and L22, methylation of 23S rRNA by methyltransferases, decreased uptake of drugs via increased extrusion by efflux pumps and decreased permeability of the outer membrane, and inactivation of drugs by modifying enzymes [10]. Several mechanisms of azithromycin resistance have been found in Salmonella, including modification of the drug by *Mph* (A), a macrolide 2′-phosphotransferase [13], methylation of 23S rRNA by *ErmB* and *Erm42*, rRNA adenine N-6-methyltransferases [16], and increased drug extrusion by an *AcrAB-TolC* efflux pump that has an *R717* mutation in *AcrB*. The resistance determinants can be carried by plasmids, transposons, including integrative and conjugative elements and chromosomes [17].

MATERIALS AND METHODS

Study Area

The study area was general hospital Keffi. Keffi is approximately 68km from Abuja, the Federal Capital Territory and 128km from Lafia, the Capital of Nasarawa state Keffi is located between latitude 8°5 N of the equator and longitude 7°8 E and situated on an altitude of 850m above sea level [18].

Ethical Approval

Ethical approval was obtained from the ethical committee of Nasarawa State hospital Management Board Lafia.

Sample Size Determination

The sample size was calculated manually using the formula below

$N=Z^2P\sum/d^2$

Where: N= desired sample size (when the population >10,000); Z= standard normal deviate, usually set at 1.96, which usually correspond to 95% confidence level; P= proportion in the target population, set at 50% (0.5) d= tolerated margin of error.

The proportion was estimated as p< (0.5) and q< (0.5) for non-infection confidence estimated used was 95% (≥1.5) confidence interval with degree of accuracy of d (0.05). The designed effect of 1 was used. The sample size was obtained

N= (1.16)2× 0.5× 0.5÷ (0.05)2

=0.9404÷0.0025 = **312**

Sample Collection

Three hundred and twelve (312) stool samples was collect from patients attending selected General Hospital Nasarawa state, into a sterile screw sample bottle. The samples were transported to microbiology laboratory Nasarawa State University Keffi for isolation of *Salmonella* typhi.

Culture and Isolation of *Salmonella* typhi

A loopful of the stool sample was transferred into 5ml Selenite F broth (Oxoid, UK) and incubated at 35^0C for 18-24 hours. All the tubes showing turbidity after incubation was bacteriologically cultured on *Salmonella-Shigella* agar (SSA) (Oxoid, UK) and Bismuth Sulfite agar for the selective isolation of *Salmonella* species. The plates were incubated at 37ºC for 24 hours. After incubation, the plates was observed for growth and subsequently stored in a slant bottle for further use.

Identification of the Isolates

Representative colonies were chosen from each of the cultured plate on the bases of their colonial and morphological similarities. Pure bacterial colonies were identified using Gram staining reaction and biochemical tests according to the methods of Cheesbrough [19].

Gram Staining Examination

The Gram staining technique was carried out as described by Cheesbrough [19]; A small portion of culture organism was transferred onto a clean grease-free glass slide, and emulsified in a drop of distilled water until a thin homogeneous film is obtained, then the wire loop was re-sterilized and the thin homogeneous film was allowed to air-dry and heat-fixed by passing through the flame. The slide was then flooded with crystal violet for 1 minute and then rinsed with distilled water. The stain was again flooded with Lugol's iodine for 1 minute and rinsed with distilled water and then decolorized rapidly with acetone alcohol until no more colour appeared to flow from preparation and rinsed appropriately with distilled water. The stain was then counter-stained with neutral red for 1 minute and rinsed with distilled water and allowed to air dry and viewed microscopically using x100 oil immersion objective.

Biochemical Tests

The following biochemical tests was carried out on the suspected *Salmonella* species isolates: Catalase test, Indole, Methyl red, Vorges-Proskauer tests, Nitrate reduction, Urease production, Citrate utilisation, and glucose fermentation tests.

Indole Test

The Indole Test for the suspected organism was carried out as described by Cheesbrough [19]. A colony of the organism from culture plate was inoculated unto 5ml tryptone broth and incubated at 37ºC for 24 hours. After which a few drops of Kovac's reagent was added to the overnight tryptone broth culture, and shaken. A positive reaction is indicated by the development of red ring colour in the reagent layer above the broth within 10 minutes observed. *Salmonella* species is negative for this reaction.

Methyl Red/Vorges-Proskauer Test

The Methyl Red Test for the suspected organism was carried out as described by Cheesbrough [19]. A pure culture of test organism was inoculated in to MR-VP medium and incubated at 37ºC for 72 hrs after which the culture was divided in to two portion. To the first portion three drops of methyl red was added and formation of red colour was indicative of methyl red positive. To the second portion 10 drops of 40% KOH (Potassium hydroxide) was added followed by four drops of alpha-napthol added and observed for 30 minutes. Formation of pink/red colour indicates Voges-Proskauer positive and formation of yellow colour indicates Voges-Proskauer negative. *Salmonella* species is positive for methyl red and negative for Vorges-Proskauer.

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Citrate Utilisation Test

The Citrate Utilization Test for the suspected organism was carried out as described by Cheesbrough [19]. A pure culture of the organism was inoculated as a single streak on the slant surface of citrate agar and was incubated at 37^{o}C for 24 hours, blue colour on the medium indicated the presence of alkaline products and it is therefore positive, while green colour is negative. *Salmonella* species is positive for citrate utilization.

Catalase Test

Catalase test was performed as described by Cheesbough [19]. A pure colony of the organism was streaked aseptically on Nutrient agar slant and incubated at 37^{o}C for 24hours. Three drops of Hydrogen peroxide (H_2O_2) was added to the slant and observed for bubbling gas. *Salmonella* species is positive for this test.

Urease Test

This was performed by inoculating the organism into Urea broth and incubated at 37^{o}C for 24hours. Intense pink colour indicates positive, otherwise, it is negative [19]. *Salmonella* species are negative for urease production.

Determination of Antibiotic Susceptibility of the *Salmonella* Typhi

The antibiotic susceptibility of the *Salmonella* Typhi isolates was determined using the Kirby-Bauer disk diffusion method. Briefly, a suspension of each isolate was prepared in peptone water to match 0.5 McFarland turbidity standards. The standardized inoculums of each isolate was then inoculated in triplicates onto the surfaces of plain Mueller-Hinton agar plates and ciprofloxacin (5µg), gentamicin (10µg), Amoxicillin/Clavulanic acid (30µg), ampicillin (30µg), azithromycin (30µg), cefuroxime (30µg), chloramphenicol (30µg), ciprofloxacin (10µg), ofloxacin (10µg) and ceftriaxone (30µg) discs was placed aseptically and incubated at 37°C for 24 hours. The zones of inhibition was measured and compared to that of the Clinical and Laboratory Standards Institute (CLSI) guidelines 2014.

Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR Index was determined according to the method of Bauer. From the result of the antibiotic susceptibility test, MARI was calculated using the following formula:

$$\text{MAR Index} = \frac{\text{No of antibiotics to which isolate is resistant}}{\text{Total classes of antibiotics No tested}}$$

Statistical Analysis

Statistical analyses was carried out using SPSS version 21.0. Differences by the chi-square (χ^2) test was considered significant, if $P< 0.05$ (CI).

RESULTS

Isolation and Identification of *Salmonella* Typhi

The cultural, morphological and biochemical characteristics of *Salmonella* Typhi from stool of patients attending selected general hospital in Nasarawa state, Nigeria is as shown in Table 4.1. Colourless colonies on DCA with black centre and black metallic sheen on BSA which were Gram negative, rod shape, positive for TSI, glucose, maltose and many others were identified as *S.* Typhi as shown in Table 1.

Occurrence of *Salmonella* Typhi

Out of 316 stool samples of patients attending selected general hospital in Nasarawa state. The occurrence of *S.* Typhi was 38(12.0%). The occurrence of isolates in relation to selected hospitals, gender and age of patients is as shown in Table 2, 3 and 4.

The occurrence of the isolates in relation to the hospital was high in Nasarawa general hospital (19.3%) followed by Karu general hospital (14.2%), Garaku general hospital (13.5 %), Maraba general hospital (11.1%) but low in Keffi general hospital (6.5%) as shown in table 4.2.

The occurrence of the isolates in relation to the gender of patient's was high in female (13.4%) then in male (10.1 %) as shown in Table 3.

The occurrence of the isolates in relation to age was high in 21-30 yrs (21.4%) followed by 11-20 yrs (13.3%), 31-40 yrs (9.7%), >50 yrs (8.1%) but low in 41-50 yrs (7.6 %) as shown in Table 4. The differences in the occurrence of the isolates in relation to the hospital were statistically significant ($P< 0.05$) but in relation to gender and age, the differences in the occurrence of isolates were statistically insignificant ($P> 0.05$).

Table 1: Cultural, Morphological and Biochemical Characteristics of *Salmonella* Typhi from stool of Patients attending selected general hospital in Nasarawa state, Nigeria

Cultural Characteristics	Morphological Characteristics		Biochemical Characteristics														Inference
	Gram stain	Mophology	Oxd	Ur	Ct	TSI	Ind	Mr	VP	Glu	Lac	Mal	Mann	Suc	Xyl	Lys	
Colourless colonies on DCA and black metallic sheen on BSA	-	rod shape	-	-	-	+	-	-	-	+	-	+	+	-	+	+	*S.* Typhi

DCA=Deoxycholate agar; BSA= Bismuth sulphide agar; -=Negative; +=positive; Oxd=Oxidase; Ur=Urease; Ct=Citrate; TSI=Triple Sugar Iron; Mr=Methyl red; VP=Voges-Proskauer; Glu=Glucose; Lac=Lactose; Mal=Maltose; Mann=Mannitol; Suc=Sucrose; Xyl=Xylose; Lys=Lysin

Table 2: Occurrence of *Salmonella* Typhi from stool of Patients attending selected general hospital, in relation to facilities

Health facilities	No. of samples	No. of (%) *S.* Typhi
NASG	31	6 (19.3)
MABG	72	8(11.1)
GRG	81	11(13.5)
KEG	76	5(6.5)
KAG	56	8(14.2)
Total	316	38(12.0)

NASG= Nasarawa general hospital, MABG= Maraba general hospital, GRG= Garaku general hospital, KEG= Keffi general hospital and KAG= Karu general hospital

Table 3: Occurrence of *Salmonella* Typhi from stool of Patients attending selected general hospital in Nasarawa state

Gender	No. of samples	No. of (%) *S.* Typhi
Male	138	14(10.1)
Female	178	24(13.4)
Total	316	38(19.7)

$\chi 2 = 0.0471$ $P_{value}=0.8282$

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Table 4: Occurrence of *Salmonella* Typhi from Stool of Patients Attending selected general hospital in Nasarawa state, in relation to age

Age group (Yrs.)	No.of samples	No. of (%) *S.* Typhi
11-20	67	9(13.3)
21-30	56	12 (21.4)
31-40	82	8 (9.7)
41-50	52	4 (7.6)
>50	61	5 (8.1)
Total	316	38 (12.0)

$\chi 2 = 1.5015$ $P_{value}=0.8264$

Antibiotic Resistance of *Salmonella* Typhi

The antibiotic resistance of *S. enterica* from stool of patients attending general hospital facilities in Nasarawa state, Nigeria is shown in Table 4.5. The isolates were highly resistant to tetracycline (81.5 %) followed by co-trimoxazole (76.3 %), ceftazidime (39.4 %) amoxicillin/clavulanic acid, netillin, levofloxacin (34.2 %) but less resistant to cefurazime (28.9%), ciprofloxacin (15.7%) azitharmicine (10.5%) and gentamicin (13.1%) respectively as shown in Table 4.5.

Antibiotic Resistance Profile

The antibiotic resistance profile of *S. enterica* isolates is as shown in Table 4.6. The commonest resistance profile in the isolates were; CTX-CAZ-AMC-TE-CTX-CAZ-S-SXT-AMC-NA-C-TE and CTX-CAZ-S-SXT-AMC-NA-CN-CIP-C-TE with the percentage occurrence of 12.8% respectively as shown in Table 4.6.

Multiple Antibiotic Resistance (MAR) Index

The MAR index of *S.* Typhi isolates is shown in Table 4.7. All MAR isolates with MAR index of ≥0.4 and the commonest MAR index in the isolates was 0.8 (33.3%) as shown in Table 4.7.

Table 5: Antibiotic Resistance of *Salmonella* Typhi from stool of Patients attending selected general hospital in Nasarawa state

Antibiotics	Disc content (µg)	Resistance (%) (n=38)
Azitharmicine	30	4(10.5)
Amoxicillin/clavulanic acid	30	13 (34.2)
Ceftazidime	30	15 (39.4)
Cefurazime	30	11 (28.9)
Ciprofloxacin	5	6 (15.7)
Co-trimoxazole	25	29 (76.3)
Gentamicin	10	5 (13.1)
Levofloxacin	5	13 (34.2)
Netillin	30	13 (34.2)
Ofloxacin	5	8 (21.0)
Tetracycline	30	31 (81.5)

Table 6: Antibiotic Resistance Profile of *Salmonella* Typhi from Stool of Patients attending selected general hospital in Nasarawa state

Antibiotic resistance profile	Frequency (%)(n=38)
CTX,CAZ,AMC,TE	5 (12.8)
CTX,CAZ,AMC,OF,TE	2 (5.1)
CTX,CAZ,AMC,CIP,AZ	1 (2.6)

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CTX,CAZ,AMC,LE,TE	3 (7.7)
CTX,CAZ,OF,NE,AMC,TE	2 (5.1)
CXT,CAZ,OF,AMC,NE,CIP,TE	1 (2.6)
CXT,CAZ,OF,COT,AMC,NE,AZ	3 (7.7)
CTX,CAZ,LE,COT,AMC,C,OF,TE	4(10.3)
CTX,CAZ,COT,AMC,NE,CIP,C,TE	4 (10.3)
CXT,CAZ,LE,COT,AMC,CIP,C,TE	5 (12.8)
CXT,CAZ,OF,COT,AMC,LE,CN,C,AZ	2 (5.1)
CXT,CAZ,LE,COT,AMC,NE,CN,CIP,TE	2 (5.1)
CTX, CAZ,LE,COT,AMC,NE,CN,CIP,TE	5 (12.8)

CTX= Ceftaxime; CAZ= Cefdtazidime; AMC=Amoxycillin/Clavulanic acid; TE= Tetracycline, CIP= Ciprofloxacin; OF=Ofloxacin; AZ= Azitharmicine, CN= Gentamicin, COT= Co-trimoxazole; LE= Levofloxacin

Table 7: Multiple Antibiotics Resistance (MAR) Index of *Salmonella* Typhi from Stool of Patients attending selected general hospital in Nasarawa state

No. of antibiotic resistance to (a)	No.of antibiotics tested (b)	MAR index (a/b)	Frequency (%) (n=38)
10	10	1.0	5 (12.8)
9	10	0.9	4 (10.3)
8	10	0.8	12 (33.3)
7	10	0.7	4 (10.3)
6	10	0.6	5 (12.8)
5	10	0.5	3 (7.7)
4	10	0.4	5 (10.3)
3	10	0.3	0 (0.0)
2	10	0.2	0 (0.0)
1	10	0.1	0 (0.0)

DISCUSSION OF FINDINGS

The isolation of *S.* Typhi from stool of patients attending selected general hospital in Nasarawa state was not surprising and this finding is an indication that the bacteria may be responsible for typhoid fever in suspected typhoid patients in the study area. The percentage occurrence of the isolates observed in the study area was less than 35.7%, 75.0%, and 72.5% reported by Okoro *et al*. [20], Amsalu *et al*. [21], and Ohanu [22] but higher than 3.2% and 14.6% reported by Inusa *et al*. [23]. and Mohammad [24]. Our findings shows that the occurrence of the isolates was high in female than the male patients and this is not in agreement with the study conducted by Bobai *et al*. [25], who reported high occurrence of the isolates in male than the female . The occurrence of the isolates in female and male patients observed in this study was less than 38.6% and 61.4% reported by Bobai *et al*. [25] but higher than 2.6% and 23% reported by Maharjan *et al*. [26]. The occurrence of the isolates in relation to age was high in 21-30 years and this finding is also different from the study conducted by Bobai *et al*. [25] who reported high percentage occurrence of the isolates in ≤10 years (33.2%). The prevalence of the isolates in relation to age and gender of patients observed in this study were statistically insignificant respectively and this implies that the gender and age of the patients attending the health facilities in the study centers does not affect infection rate. The high resistance of the isolates to antibiotics namely; tetracycline (81.5 %), co-trimoxazole (76.3 %), ceftazidime (39.4 %) observed in this study was expected and this may be due to abuse or inappropriate use of the antibiotics for treatment of bacteria related infection. The percentage resistance of the isolates to amoxicillin/clavulanic acid and ceftazidime observed in this study was different from 100.0% resistance to amoxicillin/clavulanic acid, ceftazidime also the percentage resistance to co-trimoxazole and nalidixic acid was less than 88.8% as described by Inusa *et al*. [23] in Bauchi metropolis. The resistance of the isolates to tetracycline in this study (81.5 %) was higher than 77.14% as described by Mohammad, [24] in Chattogram, Bangladesh.

The low resistance of the isolates to cefurazime (28.9%), ciprofloxacin (15.7%) azitharmicine (10.5%) and gentamicin (13.1%) observed in this study was not in agreement with the study conducted by Mohammad, [24] in Chattogram who reported high resistance of the isolates to cefurazime (65.17%) and ciprofloxacin (74.14%). The low resistance of the isolates to the antibiotics mentioned is an indication that the antibiotics may be effective for treatment of typhoidal *Salmonella* and may not have been misused or abused in the study area. The percentage resistance of the isolates to cefurazime (28.6%) and ciprofloxacin (15.7%) observed in this study was not in agreement with 0.0% and 35.0% reported by Maharjan *et al*.[26]. The spread of typhoidal *Salmonella* most times occurred through eating of foods and drinking of water that is contaminated with fecal matter.

CONCLUSION

In this study *Salmonella* typhi were isolated from stool of patients attending selected general hospital in the study area. It was observed that the *Salmonella* typhi isolated was high in Nasarawa general hospital but low in Keffi general hospital. It was also recorded that the occurrence was high among female than male. The isolates were more resistant to commonly use antibiotics in treatment of infection caused by *Salmonella* typhi.

Consent

All authors declare that 'written informed consent was obtained from the patient.

Ethical Approval

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Competing Interests

Authors have declared that no competing interests exist.

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