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Original Article

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Beta–lactamase production and conjugative ability of multidrug resistant *Salmonella Typhi* in Southern Benue, Nigeria

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ABSTRACT

Objective: To examine multidrug resistant (MDR) *Salmonella (S.) Typhi* isolates obtained from the stool samples of patients attending secondary health centers in Southern Benue, Nigeria, for extended spectrum beta-lactamase (ESBL) production and conjugative ability.

Methods: The double disc synergy test was used to screen for ESBL production in MDR isolates. The ability of *S. Typhi* to transfer resistance gene was determined by conjugation experiments using standard protocols, with slight modifications. *Escherichia (E.) coli* ATCC 25922 was used as the recipient organism.

Result: The donor *S. Typhi* isolates showed resistance to all the classes of antibiotics except aminoglycosides and carbapenems. All isolates except TKP 106, APA 55, APA 78 and OGB 106 were positive for ESBL production. Reduction in the zones of inhibition of *E. coli* after incubation with *S. Typhi* showed evidence of conjugation and transfer of antibiotic resistance from *S. Typhi* to *E. coli*.

Conclusions: The presence of ESBL-producing *S. Typhi* capable of conjugation poses a significant risk to the treatment and management of typhoid fever, which highlights the need for the regulation of antibiotic use through relevant policies and strategies that aim at halting the spread of MDR and ESBL-producing *S. Typhi* in such rural settings.

KEYWORDS: *Salmonella Typhi*; Antimicrobial resistance; Plasmid; Multidrug resistance; Extended spectrum beta-lactamase

1. Introduction

The control of infectious diseases is seriously threatened by the steady increase in antimicrobial resistant disease agents. The emergence of cephalosporin-resistant strains of *Salmonella (S.)* enterica serovar *Typhi* is a cause of concern in the management of enteric fever. *S. Typhi* causes typhoid fever, a disease associated with high cases of mortality and endemic in in most developing countries of the world[1,2], particularly in sub-Saharan Africa and South Asia[3,4]. The disease prevalence in these economically disadvantaged climes is made worse by poverty, poor infrastructure, poor sanitation and limited access to portable water[4,5]. The

Significance

Salmonella (S.) Typhi infection is endemic in developing countries. Recent reports have shown an increasing trend of multidrug resistant *S. Typhi* strains. This work further shows that ESBL-producing *S. Typhi* strains with ability to transfer resistance plasmids are in circulation within the study area. This finding exposes the significant risk posed to the treatment and management of typhoid fever, and the need for the regulation of antibiotic usage.

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use of antibiotics and public health management strategies have resulted in effective control of typhoid fever in some parts of the world. Currently, however, previously effective antibiotic treatment regimens are reportedly beginning to lose potency against *S. Typhi*, due to the rise of multidrug resistant (MDR) strains. This trend has further compounded the challenges being faced with disease treatment and management in patients[1,5].

The β-lactam antibiotics, including third and fourth generations of cephalosporins have been of immense help in the effective and timely treatment of infections in humans and animals[6,7]. Researchers have, however, reported the emergence of bacterial resistance to cephalosporins[1,8]. Resistance to antibiotics in *Salmonella* serovars, including Heidelberg from humans, poultry, and poultry meat, has also been reported, all of them presenting diverse MDR patterns[9-12]. This observation calls for serious concern, due to its impact on the treatment and management of enteric fever. Earlier reports indicated that cephalosporin resistance in *Salmonella* species was mainly due to the production of extended-spectrum β-lactamases(ESBLs), the majority of which are derivatives of TEM, SHV, and CTX-M β-lactamase families in *S. Typhi*[11-14]. Among the Enterobacteriaceae, β-lactam resistance was reported due to enzymatic inhibition[1,11,14].

Antibiotic resistance is highly influenced by horizontal gene transfer (HGT) between bacteria species, which is made possible by mobile genetic elements such as transposons, phages and plasmids through the processes of transformation, transduction and conjugation respectively[15-17]. The ease with which plasmids bearing resistance markers are transferred between bacteria is a major clinical challenge of epidemiological importance, especially because it is a common occurrence among Enterobacteriaceae. As a noneligible factor in the spread of antibiotic resistance among bacteria[5,15,18], plasmids that code for antibiotic resistance are mostly acquired from the environment through HGT, which is quite common in Gram-negative bacteria[19-21]. Since plasmids are not essential for bacterial life, recombination events in these molecules are more frequent than in chromosomes[22,23].

The global problem of antibiotic resistance is particularly pressing in developing countries where the infectious disease burden is high[13,24,25]. This is coupled with cost constraints which prevent the administration of newer and more expensive therapeutic agents at appropriate, effective doses[1,26]. Unfortunately, antibiotic administration at sub-therapeutic doses could result in selective pressure in favour of antibiotic resistant pathogens[27], and such practices could be prevalent particularly in economically disadvantaged societies. Antibiotic treatment options for diseases become limited when MDR pathogens spread among human and animal populations through HGT, which could result in increased disease burden, treatment cost and length of hospitalization[26]. Recently, we reported the existence of MDR *S. Typhi* in Southern Benue, Nigeria[8,26]. It is important to understand if these plasmids are transferred within a community of bacteria, as this would assist in determining the risk of resistance genes dissemination, particularly in such resource constrained societies. Also, preliminary characterization of ESBL-producing *S. Typhi* strains previously identified by culture and phenotypic characteristics in such a setting[28] will broaden existing knowledge about the extent of their occurrence within the rural communities of Southern Benue, and their conjugative ability. This knowledge will equally enable an understanding of the differences in gene content and transfer mechanisms, to accurately determine the impact of plasmids on the dissemination and persistence of antibiotic resistance genes.

2. Materials and methods

2.1. Bacterial strains and antibiotic resistance

S. Typhi strains were isolated from stool samples of patients attending secondary health centres in Southern Benue. Inoculation was first performed into Selenite broth base (CM0395; Oxoid, UK) which was incubated for 18–24 h, and a loopful was streaked on Salmonella Shigella agar (CM 0099; Oxoid, UK) and Xylose Lysine Deoxycholate agar (CM 0469; Oxoid, UK). Pure colonies were obtained by subculturing suspected colonies on Bismuth Sulfite agar (CM 0201; Oxoid, UK) for 18–24 h[28]. The bacterial strains were identified using conventional culture and biochemical tests such as Gram reaction, motility, catalase, indole, oxidase, citrate utilization and Triple sugar iron tests[28]. A total of 447 *S. Typhi* isolates were isolated and identified from 1 022 collected samples. *Escherichia (E.) coli* ATCC 25922 was obtained from archived bacterial cultures at the National Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria and was used as the recipient organism. Upon receipt, the *E. coli* was sub-cultured in sterile nutrient broth (CM0067; Oxoid, UK) as well as on MacConkey agar (CM0007; Oxoid, UK) and Eosine Methylene Blue agar (CM0069; Oxoid, UK) for 18–24 h at 37 ℃. The MacConkey agar plates were examined for lactose fermenting properties of *E. coli* while the EMBA plate was examined for the presence of green metallic sheen as preliminary confirmation of *E. coli*.

2.2. Antibiotic susceptibility tests

To determine the MDR status of the *S. Typhi* isolates, antibiotic susceptibility test was performed using the disc diffusion method. The discs were obtained from Oxoid, United Kingdom. Overnight cultures of each isolate were inoculated in phosphate buffered saline and the turbidity adjusted to match 0.5 McFarland. The standardized cultures were each streaked on Mueller-Hinton agar surface, following which antibiotic impregnated discs were placed, namely: ceftazidime (30 μg), chloramphenicol (30 μg), gentamicin (30 μg), amoxicillin-clavulanate (30 μg), amoxicillin (10 μg), ciprofloxacin (5 μg), ceftriaxone (30 μg), azithromycin (15 μg), imipenem (10 μg) and sulphamethoxazole/trimethoprim (25 μg). After 18 h of incubation, the resulting inhibition zones were interpreted following the Clinical Laboratory Standard Institute (CLSI) interpretative zones criteria[29].

Purified *E. coli* strains were also tested for their susceptibility to ciprofloxacin, amoxycillin, amoxicillin-clavulanate and ceftriaxone. The resulting zones of inhibition of the test antibiotics against the susceptible *E. coli* were determined using Kirby Baur Disc Diffusion method. Results were interpreted using the CLSI interpretative zones criteria[29].

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2.3. Confirmation of ESBL production in S. Typhi isolates

The double disc synergy test was used to screen for ESBL production in the isolates that exhibited multi-drug resistance following previously reported protocol[30]. Sub-culture of each randomly selected MDR *S. Typhi* isolate was made on Mueller-Hinton agar and exposed to antibiotic discs of ceftazidime (30 µg) and ceftriaxone (30 µg) placed at 15 cm equidistance to a central disc of amoxicillin-clavulanate (30 µg). The inoculated plates were then incubated at 37 ℃ for 18-24 h. An isolate was confirmed as ESBL producer if the zone of inhibition around any of the antibiotic disc increased towards the amoxicillin/clavulanic acid disc. It was also ESBL producer if a difference of 5 mm was observed between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disc.

2.4. Determination of S. Typhi conjugative ability

The ability to transfer resistance plasmid by *S. Typhi* was determined by conjugation experiments following standard protocols as previously reported with some modifications[19,22]. Ciprofloxacin, amoxycillin, amoxicillin-cluvanate and ceftriaxone were used as selection agents instead of ampicillin and tetracycline. Purified susceptible *E. coli* isolates (recipient) as well as MDR *S. Typhi* isolates (donor) were separately sub-cultured into sterile nutrient broth and incubated at 37 ℃ for 18–24 h. The overnight cultures of the donor *S. Typhi* and the recipient *E. coli* were mixed in a ratio of 10:1 (*S. Typhi: E. coli*) in 50 mL of sterile nutrient broth and incubated at 37 ℃ for 18–24 h. A loopful of the resulting trans-conjugant from the admixtures was sub-cultured onto MacConkey agar and eosine methylene blue agar, plates and incubated at 37 ℃ for for 18-24 h to select surviving *E. coli* cells. The colonies of *E. coli* identified were then sub-cultured onto nutrient agar slants and the antibiotic susceptibility profiles of the trans-conjugants determined using the disc diffusion method. The resulting inhibition zones were interpreted using the CLSI interpretative zones criteria[29].

3. Results

The MDR status of the donor *S. Typhi* before conjugation is presented in Table 1. Out of 447 *S. Typhi* isolates, 64.0% (286/447) showed resistance to at least one antibiotic class, while 36.0% (161/447) isolates were susceptible to the tested antibiotics. Out of the 286 resistant *S. Typhi* isolates, 42.7% (122/286) were determined to be MDR strains, while 57.3 % (164/286) where non-MDR strains. Of the 122 MDR strains, 54.1% (66/122) were resistant to three classes of antibiotics, 17.2% (21/122) to four classes and 28.7% (35/122) ⩾5 classes of antibiotics respectively.

Production of ESBL in the *S. Typhi* isolates was determined using the double disc synergy test. As shown in Table 2, all randomly selected isolates except TKP 106, APA 55, APA 78 and OGB 106 were positive for ESBL production. While all the other isolates were sensitive to at least one antibiotic, isolates TKP 106, APA 55, APA 78 and OGB 106 were all resistant to the Ceftazidime, ceftriaxone and amoxicillin-clavulanate used for the double disc synergy test.

Table 1. Occurrence of MDR in *Salmonella Typhi* isolates.

No. of antibiotic classes	Resistance frequency	Percentage occurrence (%)
＜3 (Non-MDR)	164	57.3*
3	66	54.1
4	21	17.2
⩾5	35	28.7
Total MDR	122	100

* Percentage of non-MDR out of the 286 *Salmonella Typhi* isolates that showed resistance to at least one antibiotic class; 122 isolates showed resistance to at least three antibiotic classes.

Table 2. Determination of extended spectrum beta-lactamase (ESBL) production in the Salmonella typhi Isolates using the double disc synergy test.

Isolate	CRO	CFT	AMC	ESBL result
TKP 49	R	R	S	+
TKP 106	R	R	R	-
OHM 22	S	R	S	+
OHM 46	S	S	S	+
OJU 51	R	R	S	+
OJU 91	R	R	S	+
OBI 63	R	S	S	+
OBI 66	R	S	S	+
APA 55	R	R	R	-
APA 78	R	R	R	-
GTU 5	S	R	S	+
GTU 52	R	S	S	+
ADO 7	R	R	S	+
ADO 27	R	R	S	+
OKP 86	R	R	S	+
OKP 124	R	R	S	+
OGB 106	R	R	R	-
OGB 117	R	S	S	+

Note: R = Resistant; S = Susceptible; + = ESBL producer; - = non-ESBL producer; CRO = Ceftriaxone; CFT = Ceftaxidime; AMC = Amoxycillin-clavulanic acid.

The inhibition zones (mm) of ciprofloxacin, amoxicillin, ceftriaxone and amoxicillin-clavulanic acid against the recipient *E.coli* before and after conjugation experiments with *S. Typhi* is presented in Table 3. A reduction in the zones of inhibition of recipient *E. coli* strains after incubation with *S. Typhi* was observed. Significant reduction in inhibition diameter of recipient *E. coli* from 18 mm (ciprofloxacin), 19 mm (amoxicillin), 20 mm (ceftriaxone) and 22 mm (amoxicillin-clavulanic acid) to less than 10 mm was observed after conjugation with donor *S. Typhi*. Four recipient *E. coli* isolates (OJU 31, OJU 72, OKP 91 and OKP 116) became completely resistant to ceftriaxone after conjugation with the donor MDR *S. Typhi*. Similarly, recipient *E. coli* isolates OJU 46, ADO 17 and OGB 39 acquired total resistance to amoxicillin-clavulanic acid after conjugation with donor MDR *S. Typhi* isolates.

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Table 3. Inhibition zones (mm) of commonly used antibiotics against *Escherichia coli* ATCC25922 [recipient or female (F-)] organism before and after conjugation with *Salmonella Typhi.*

Samples	Inhibition zone (mm)							
	Ciprofloxacin		Amoxicillin		Ceftriaxone		Amoxicillin- clavulanic acid	
	Before	After	Before	After	Before	After	Before	After
TKP 32	19	8	18	9	20	7	22	8
TKP135	19	6	18	7	20	8	22	6
TKP148	19	7	18	8	20	8	22	8
OHM19	19	5	18	6	20	7	22	7
OHM33	19	8	18	8	20	9	22	6
OHM48	19	7	18	8	20	8	22	5
OJU31	19	8	18	9	20	0	22	2
OJU46	19	7	18	5	20	3	22	0
OJU72	19	8	18	4	20	0	22	3
OBI25	19	5	18	4	20	9	22	3
OBI44	19	8	18	7	20	8	22	4
OBI65	19	6	18	5	20	8	22	3
APA68	19	5	18	6	20	5	22	4
APA66	19	5	18	5	20	6	22	4
APA78	19	7	18	8	20	9	22	5
GTU8	19	5	18	8	20	4	22	3
GTU52	19	7	18	8	20	4	22	5
GTU82	19	7	18	5	20	6	22	3
ADO17	19	6	18	6	20	4	22	0
ADO46	19	8	18	6	20	5	22	3
ADO57	19	7	18	7	20	5	22	4
OKP85	19	6	18	5	20	6	22	3
OKP91	19	6	18	4	20	0	22	2
OKP116	19	7	18	8	20	0	22	2
OGB39	19	6	18	7	20	4	22	0
OGB85	19	7	18	7	20	3	22	3
OGB95	19	6	18	6	20	4	22	2

4. Discussion

Multi-drug resistance is defined as simultaneous resistance to three or more antibiotic classes by a microorganism[31,32]. Our earlier report had shown a prevalence of 64.0% resistant *S. Typhi* out of 447 isolates in the study area[8]. The high percentage of resistant isolates which were MDR further confirms previous reports about the increasing incidence of antibiotic resistance in pathogenic bacteria in Nigeria[33,34], and other parts of the world[35-37], particularly resistance to more than three antibiotic classes[2,38]. For instance, it has been reported that 40.0%–60.0% of *Staphylococcus aureus* isolates obtained from various United States hospitals showed resistance to methicillin, carbapenems and vancomycin[34]. The trend of increasing resistance to previously effective antibiotics has also been reported in species of *Salmonella* between 2016 and 2020 in different countries of the world[40]. MDR bacteria have been associated with cases of nosocomial and community-acquired infections, which in turn affect patient outcomes, with consequent increase in mortality and morbidity[41]. Substantial increase in treatment cost and length of hospitalization have also been associated with MDR strains, thereby increasing healthcare burden, in the face of declining antibiotic treatment alternatives[39,42,43]. Practices such as drug over-use and misuse, self-prescription and the lack of sufficient and qualified health personnel in rural communities like the study area, have been associated with the development of antibiotic resistance[26,44]. Being predominantly farmers engaged in both crop and animal farming, the possibility of excessive antibiotic use in livestock as growth supplements is high, and could contribute to increased antibiotic resistance[39,45]. In a recent study, we reported a possible positive reversal in the susceptibility of *S. Typhi* strains to chloramphenicol which is no longer commonly prescribed in the study area. Suspending the use of a drug over a period of time could reverse resistance trends by eliminating selective pressure[1,46]. This assertion could also perhaps, be explored in the fight against the increasing trend of antimicrobial resistance.

ESBL-producing bacteria have become a serious concern in healthcare and clinical practice lately. For instance, ESBL-producing *S. enterica* were reported as a leading cause of gastroenteritis in humans which was estimated to cause about 155 000 deaths annually[47]. Unfortunately, the detection of ESBL-producing bacterial strains is not a routine practice and is not regularly identified consequently[48,49], which brings potential danger in resource constrained environments where only routine susceptibility tests are performed in clinical settings. A report by Jernigan *et al.* indicated a 53.3% increase in the incidence of ESBL associated infections within the United States between 2012 to 2017[43]. The report further demonstrated that the increase was driven by cases that emerged from within the community. Similarly, an earlier study

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in Lagos, Nigeria, revealed the circulation of $bla_{CTX-M-1}$ ESBL *S. Typhi* strains among patients, with accompanying 71.4% of them experiencing persistent pyrexia[50]. Other studies have also reported ESBL producing *Salmonella* and other bacteria in different parts of Nigeria[51-53]. Previous reports have shown a higher prevalence of ESBL-producing bacteria pathogens in North-East Nigeria (16.7% to 82.3%) than in North-West Nigeria (12.8% to 41.2%) [49]. In this study, most of the *S. Typhi* isolates tested for ESBL production using the double disc synergy test were found to be positive. This finding could mean that ESBL strains of *S. Typhi* could be spreading within the rural communities of Southern Benue, with a high chance of reduced antibiotic efficacy, or increased treatment failure[7,10,13,26]. Information on ESBL-producing bacteria in Benue state is very scarce, and this study is the first to our knowledge, to report ESBL-producing *S. Typhi* in the entire Southern Benue. To stem the tide, it may be necessary to add ESBL testing to routine susceptibility tests, in addition to a regulation of the prescription and abuse of third generation cephalosporins among rural dwellers. As the race against MDR pathogens continues, it may be difficult to match the development of new alternative antimicrobial agents, with the rate at which MDR increases. Efforts such as the use of "nano-antibiotics" and surface engineered nanocarrier approach and systems might be of great help in tilting the balance in favour of more effective antibiotics in healthcare delivery[54].

Conjugation experiments performed in this study showed that resistant plasmids harboured by *S. Typhi* were transferable. This is because the hitherto susceptible *E. coli* ATCC 25922 acquired some levels of resistance demonstrated by a reduced zone of inhibition after conjugation with the MDR *S. Typhi*. This result corroborates the earlier findings of Hradecka *et al*.[22], Waleed *et al*.[20] and McMillan *et al*.[21] on HGT by salmonellae. The ability of bacteria to acquire resistance from the environment pose a great threat to antibiotic therapy which unfortunately is the mainstay for the treatment of typhoid fever[1,21,24]. Beside plasmid exchange through HGT, mobile genetic elements also aid the community spread of antimicrobial resistance. Certain reports have demonstrated association between resistance to certain antibiotics and the presence of integrons[55,56]. Since conjugative plasmids are sometimes capable of self-transmission[21], they can contribute significantly to the spread of antimicrobial resistance in a community. Hence, the observance of HGT from MDR *S. Typhi* to recipient *E. coli* portends serious issues in the treatment of diseases in the study area and such similar settings. Ciprofloxacin and ceftriaxone are two principal antibiotics used for the treatment of *Salmonella* infections in recent times. Interestingly, some of the recipient cells used in this study developed complete resistance to ceftriaxone and amoxycillin-clavulanic acid after the conjugation experiment, suggesting that the transferred plasmid exhibited potent resistance characteristics in the recipient cell. Although *S. Typhi* was the donor cell in this study, it has also been suggested that genetic transposition events could facilitate the acquisition of antimicrobial resistance genes by the virulence plasmids of *Salmonella*, thereby transferring resistance traits from other strains to species of *Salmonella* during conjugation. Resistance plasmids that aid resistance to Azithromycin in *S. Typhimurium* has also been reported, a development which further lessens the options available for treating salmonella infections[57]. Interestingly, this phenomenon of highly transmissible plasmid HGT has also been reported among *Salmonella* strains that infect animals, highlighting the potential for zoonosis in the future[58]. The exchange of resistance genes by Gram-negative bacteria of different genera has earlier been reported as a form of evolutionary rescue that can transform an ESBL-susceptible bacterium into a resistant strain[59], and this could sometimes remain unchanged even at elevated antibiotic doses[59,60]. The application of CRISPR-elements targeted at β-lactamase producing pathogens during or before antibiotic treatment in the fight against MDR pathogens, has been advocated to reduce the acquisition of resistance genes by pathogens[59].

Although this study is the first, to the best of our knowledge, to report ESBL-producing *S. Typhi* in the Southern Benue zone, further work is required, using molecular tools and bioinformatics, to delineate the predominant resistance plasmids and genes that are predominant in the study area. This will aid proper understanding of the MDR *S. Typhi* crisis and appropriately generate data that would guide the adoption of strategies and policies for effective management.

5. Conclusions

Antibiotic resistance among pathogens is assuming a worrisome dimension globally, with attendant consequences on healthcare cost and patient outcomes. Unfortunately, the rate at which resistance to available treatment options develops appears to be faster. More worrisome is the spread of ESBL-producing bacteria such as *S. Typhi* among humans, which has rendered previously efficient antibiotics almost useless in recent times. With the ability of *S. Typhi* to engage in HGT, the risk of resistance plasmid exchange among bacteria communities of the same or different genera further compounds the increasing challenge of treating or managing an improvement in food safety and environmental practices, and the regulation of antibiotic usage in humans and animals is becoming increasingly necessary if the health of the general public must be protected from the ravaging effect of resistant pathogenic bacterial strains. Strict implementation of the antibiotic stewardship program that ensures the preservation of efficacious antibiotics through the administration of appropriate drugs, at appropriate doses, specific for a disease agent, and the discouragement of arbitrary use of broad-spectrum antibiotics in clinical settings could also help mitigate the problem of MDR. Genetic engineering can also be explored to facilitate the development of more effective antibiotics and antibiotic delivery systems for use in healthcare.

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Conflict of interest statement

The authors claim there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

Adikwu P, Umeh EU, Ogbonna IO and Ihuekwumere CC designed the experiments. Adikwu P and Obande GA analyzed the data and wrote the first draft. Adikwu P and Obande GA critically revised the article. All authors contributed to the reviewing and editing of subsequent drafts and approved the final version.

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