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Extended-spectrum Beta-lactamases Encoding Genes among *Salmonella Enterica* serovar Typhi Isolates in Patients with Typhoid Fever from four Academic Medical Centers in Lagos, Nigeria

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ABSTRACT

Background: There is scarce information about the occurrence of extended-spectrum β-lactamases (ESBLs) in *Salmonella enterica* serovar Typhi (*S. Typhi*) from patients with typhoid fever. **Objective:** To study the antimicrobial resistance and ESBL encoding genes among *S. Typhi* isolates in aforesaid patients from Lagos, Nigeria. **Methods:** *S. Typhi* isolates were collected from blood samples of typhoid fever patients from 4 academic medical centers in Lagos, Nigeria. The identification of isolates and their antibiotic susceptibility testing were performed by standard bacteriological techniques and disc diffusion method, respectively. The production of ESBLs was investigated using combination disk test (CDT) and polymerase chain reaction (PCR). **Results:** A total of 27 *S. Typhi* isolates was collected. All isolates were susceptible to imipenem and nitrofurantoin. Fifteen (55.6%) isolates were multidrug-resistant (MDR). The CDT test showed 11 (40.7%) ESBL producer isolates. However, the PCR revealed a higher occurrence rate for ESBL producers (66.7%, n = 18/27). The ESBL genes were as follows: bla_{CTX-M} (37.0%, n = 10/27), bla_{SHV} (18.5%, n = 5/27), and bla_{TEM} (44.4%, n = 12/27). All ESBL positive *S. Typhi* isolates were MDR. **Conclusions:** This study showed the emergence of ESBL-harboring *S. Typhi* in patients with typhoid fever from Nigeria. (REV INVEST CLIN. 2022;74(3):165-71)

Keywords: Antimicrobial resistance. ESBLs. Nigeria. *Salmonella enterica* serovar Typhi.

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INTRODUCTION

There are over 2500 different *Salmonella* serotypes or serovars found within two *Salmonella* species: Low socioeconomic status and poor hygiene are risk factors for *Salmonella enterica* serovar Typhi *(S. typhi)* infections[1,2]. *Salmonella* infections can be categorized as minor and major forms. Non-typhoid *Salmonella* species cause minor salmonellosis with self-limiting diarrhea and rarely bacteremia or meningitis[3,4]. While, typhoid fever caused by *S. Typhi*, presents the most serious manifestation of major salmonellosis that is characterized by fever, abdominal pain, nausea, malaise, headache, and cough[3,5].

In the absence of treatment, typhoid fever can have a fatality rate of 10–30%, but with the proper management, the rate may drop to 1–4%[3,6]. Until the 1970s, effective first-line therapies included beta-lactams such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. However, the emergence of multidrug-resistant (MDR) isolates has increasingly reduced the efficacy of these drugs[7,8]. In recent years, third-generation cephalosporins have been used in the treatment of typhoid fever, especially in cases when there are no other options available. Yet, outbreaks of cephalosporin-resistant *S. Typhi* have also been reported[7,8].

The production of β-lactamase enzymes, including extended-spectrum β-lactamase (ESBL), is a key mechanism of resistance to β-lactam antibiotics. Infections caused by ESBL-producing organisms have been on the rise in recent years. Several investigations have found that gram-negative bacteria that produce β-lactamases are recovered from both hospitalized patients and community settings[9]. Until the first decade of the 21st century, ESBL-producing *S. Typhi* strains were uncommon, occurring only occasionally in some Asian countries[10,11]. However, in recent years, the emergence of MDR ESBL-producing Salmonella species, both from human and animal sources, has raised concerns[12-14].

ESBL enzymes are capable of hydrolyzing and inhibiting broad-spectrum antimicrobials such as aztreonam, penicillins, and third-generation cephalosporins. There is a large number of ESBLs that derive from cefotaxime-hydrolyzing β-lactamase-Munich (CTX-M), Temoneira Class A extended-spectrum β-lactamase (TEM), and SHV (sulfhydryl variant of the TEM enzyme) types. Each of these enzymes has more than two hundred different variants[15,16]. *Enterobacterales* that produce ESBLs are usually present in serious infections, which affect the choice of empirical antimicrobial therapy[9].

So far, numerous studies have been conducted on the prevalence of ESBL enzymes in gram-negative bacteria from different regions of Nigeria. However, there is scarce information about their occurrence in *S. Typhi* in patients with typhoid fever. Hence, this study was aimed to evaluate the antimicrobial resistance and extended-spectrum β-lactamases encoding genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}) among *S. Typhi* isolates in patients with typhoid fever from Lagos, Nigeria.

METHODS

This study was approved by the Ethics Committee of the Lagos State University, Ojo, Lagos, Nigeria according to the Declaration of Helsinki. All patients signed an informed consent form.

Sample collection and processing

During 2015, blood samples were collected from patients suspected of having typhoid fever referred to the Microbiology divisions of four locations in Lagos, Nigeria: Lagos State University Teaching Hospital, Nigerian Institute of Medical Research, Central Health Laboratory, and Iba Primary Health Centre, Ojo. The patients recruited were both male and female 5-45 years of age. Blood samples were inoculated into brain heart infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom) in the ratio of 1:9 and incubated at 37°C for 18-24 h. After an overnight incubation, subculturing from BHI was made aseptically on MacConkey agar and *Salmonella-Shigella* agar (Oxoid, Basingstoke, United Kingdom) plates.

Bacterial identification

Positive plates were observed for colonial morphological characteristic (size, color, and texture). These discrete colonies were subjected to Gram staining for further identification of the colonies. The primary identification of *Salmonella* species was performed based on the results of a panel of standard

bacteriological tests as follows: colorless colonies on MacConkey agar, urease negative, oxidase negative, indole negative, motility positive, red slope (alkaline), and yellow (acid) butt with/out gas or H2S production on Triple Sugar Iron agar, and citrate negative[17]. The final confirmation of *S. Typhi* isolates was performed by serological serotyping using *Salmonella* polyvalent antisera (Welcome Diagnostic, London, United Kingdom) according to the manufacturer's instructions. After final confirmation, all isolates were stocked in trypticase soy broth (TSB)/glycerol (15% v/v), placed in cold box and transferred to Central Health Laboratory for antibiotic susceptibility testing (AST) and ESBL screening.

Antibiotic susceptibility testing (AST)

AST was performed on Müller-Hinton agar (MHA) according to the Clinical and Laboratory Standard Institute (CLSI) 2017 procedures against the following antibiotics: amoxicillin-clavulanate (30 µg; amoxicillin 20 µg/clavulanic acid 10 µg combination), ciprofloxacin (20 µg), gentamicin (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), cefuroxime (30 µg), ofloxacin (5 µg); cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), imipenem (10 ug), and nitrofurantoin (300 µg) (Oxoid, Basingstoke, United Kingdom)[18]. *Escherichia coli* ATCC® 25922™ was used as a quality control strain. Resistance to at least three different classes of antibiotics was considered multidrug resistance. Multiple antibiotic resistance indexes (MARI) were measured based on the ratio of an organism's resistance to antibiotics compared with how many antibiotics that organism was exposed to. MARI was calculated as a/b, where a = number of isolates which are resistant to antibiotics and b = the total number of antibiotics used. A MARI of more than 0.2 indicated that the bacterial isolates were associated with a highly contaminated environment or an environment with a high load of antibiotic use[19].

Phenotypic detection of extended-spectrum β-lactamases genes

Combination disk test (CDT) was used to screen the ESBLs in all isolates that showed reduced susceptibility or resistance to one of the following antibiotics: cefotaxime (30 µg), ceftriaxone (30 µg), and ceftazidime (30 µg) in AST stage according to the CLSI protocol[18]. Standard inoculum (0.5 McFarland) of the test isolate was cultured on MHA. The third-generation cephalosporins [cefotaxime (30 µg) and ceftriaxone (30 µg)], alone and in combination with clavulanic acid was placed 20 mm apart (center to center) on MHA plates. All plates were incubated at 35 ± 2°C for 16-18 h. If the diameter of the zone increased by 5 mm or more when third-generation cephalosporin/clavulanic acid combination was used, ESBL production was evident. *E. coli* ATCC® 25922™ and *Klebsiella pneumoniae* ATCC® 700603™ were used as the negative and positive control strains, respectively[18].

Molecular detection of extended-spectrum β-lactamases

The DNA extraction was performed using boiling method with minor changes as previously described[20]. A bacterial cell pellet was obtained by centrifuging an overnight culture of each isolate in Mueller Hinton broth at 10,000 rpm for 5 min. The pellet was suspended in 200 µL of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid). After mixing briefly on the vortex mixer, the suspension was placed on a heating block at 100°C for 10 min, followed by centrifugation at 10,000 rpm for 5 min; 100 µL of supernatant were transferred to a sterile tube and stored at −20°C until polymerase chain reaction (PCR) testing. Spectrophotometer (BIO-RAD SmartSpec™ 3000; USA) was used to determine the DNA concentration and purity.

Detection of extended-spectrum β-lactamases genes

The detection of bla_{TEM}, bla_{CTX-M}, and bla_{SHV} was done by multiplex PCR (M-PCR) using specific primers (Promega Corp, Germany) as previously published (Table 1)[21,22]. Each PCR reaction included a 20 µL volume, comprising 10 µL of ×1 PCR buffer (pH 8.3), 1.5 nm of $MgCl_2$, 200 nM each of the deoxynucleotide triphosphates, 0.5 µL of 40 pmol each of the forward and reverse primers, 1 µL of genomic DNA template (~100 ng), 1.25 U of Taq polymerase (Promega Corp, Germany), and DNA/RNA free water up to 20 µL. The PCR program (TC-312 thermal cycler, Techne, Netherlands) consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles of

Table 1. Primers used for polymerase chain reaction amplification of extended-spectrum beta-lactamases genes

Target	Primer name	Primer sequence (5'-3')	Product size (bp)	Reference
bla_{TEM}	TEM-F	TCCGCTCATGAGACAATAACC	931	21
	TEM-R	TTGGTCTGACAGTTACCAATGC		
bla_{SHV}	SHV-F	TGGTTATGCGTTATATTCGCC	868	21
	SHV-R	GGTTAGCGTTGCCAGTGCT		
bla_{CTX-M}	CTX-F	TCTTCCAGAATAAGGAATCCC	909	22
	CTX-R	CCGTTTCCGCTATTACAAAC		

DNA denaturation at 94°C for 30 s, primer annealing at 54°C for 30 s, and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 7 min was added. Five-microliter aliquots of PCR product were analyzed by gel electrophoresis with 2% agarose (Sigma-Aldrich, USA). Gels were stained with ethidium bromide at 0.5 µg/mL and visualized by UV transillumination. A 100-bp DNA ladder (Fermentas, Canada) was used as marker to extrapolate the PCR product.

RESULTS

S. Typhi isolates and antibiotic susceptibility

A total of 27 non-duplicate *S. Typhi* isolates were identified and confirmed using standard biochemical tests and serotyping method during the study period. The isolates were collected from 15 female and 12 male patients. The results of AST showed that all isolates were susceptible to imipenem and nitrofurantoin, while most isolates were resistant to trimethoprim/sulfamethoxazole (92.6%, n = 25/27). More than 50.0% of isolates (n = 17/27) were resistant to third-generation cephalosporins. In total, 17 (63.0%), 13 (48.1%), and 13 (48.1%) isolates were resistant to ceftazidime, ceftriaxone, and cefotaxime, respectively. Furthermore, 13 (48.1%) isolates were simultaneously resistant to third-generation cephalosporins. The resistance rates to other antibiotics were as follows: amoxicillin-clavulanate (18.5%), ciprofloxacin (33.3%), ofloxacin (48.1%), gentamicin (22.2%), and cefuroxime (59.3%). In total, 15 (55.6%) isolates were MDR with 12 different patterns (Table 2). MDR profiles were detected with frequencies of 13.3%, 26.7%, 13.3%, 20.0%, 13.3%, and 13.3% resistant to 8, 7, 6, 5, 4, and 3 antibiotics, respectively. The MARI indexes of *S. typhi* isolates were as follows: 0.2 (29.6%), 0.3 (7.4%), 0.4 (18.5%), 0.5 (22.2%), 0.6 (14.8%), and 0.7 (7.4%). Most isolates had MARI of 0.2.

Table 2. Multidrug resistance patterns of 15 multidrug-resistant *Salmonella enterica* serovar Typhi isolates

Antibiotic resistance patterns	Number of resistant isolates
CRX, GEN, SXT	1
CAZ, OFL, SXT	1
CRX, CRO, CFM, GEN, SXT	1
CAZ, CRX, OFL, SXT	1
CRO, OFL, CIP, SXT	1
CAZ, CRO, CFM, OFL, CIP, SXT	1
CAZ, CRX, OFL, CIP, SXT	1
CAZ, CRX, CRO, CFM, OFL, SXT	1
CAZ, CRX, GEN, OFL, SXT, AMC	1
CAZ, CRX, CRO, CFM, OFL, CIP, SXT	4
CAZ, CRX, CRO, CFM, AMC, OFL, CIP, SXT	1
CRX, CRO, CFM, AMC, GEN, OFL, CIP, SXT	1
Total	15

CAZ: ceftazidime, CRX: cefuroxime, CFM: cefotaxime, CRO: ceftriaxone, AMC: amoxicillin-clavulanate, OFL: ofloxacin, CIP: ciprofloxacin, IMP: imipenem, GEN: gentamicin, SXT: trimethoprim/sulfamethoxazole, NIT: nitrofurantoin.

Occurrence of ESBLs

The results of CDT showed that 11 (64.7%) isolates of 17 third-generation cephalosporin resistant *S.*

Typhi were ESBL producers. However, the results of M-PCR revealed that all third-generation cephalosporin resistant isolates (100.0%, n = 17/17) harbored ESBL genes. All CDT-positive isolates were also positive by M-PCR and harbored at least one ESBL gene. The occurrence rates of ESBL genes were as follows: bla_{CTX-M} (58.8%, n = 10/17), bla_{SHV} (29.4%, n = 5/17), and bla_{TEM} (70.6%, n = 12/17). The co-occurrences of ESBL genes (bla_{CTX-M} + bla_{TEM}) were detected in 52.9% (9/17) of isolates. In total, 14 (82.4%) ESBL-positive *S. Typhi* isolates were MDR.

DISCUSSION

The emergence of MDR and extensively drug-resistant *Salmonella* strains is a critical health issue that suggests that treatment of typhoid fever may become economically and practically challenging in the near future, especially in developing countries[7]. One of the causes of this high level of resistance is the transfer of antibiotic resistance genes including ESBLs among different species of Gram-negative bacteria, especially *Enterobacteriaceae*.

There are scattered reports of the prevalence of ESBL genes in *Salmonella* species of clinical origin, and the value of the theorem is such that researchers present it as a case report. In Nigeria, there is paucity of data about the ESBL-harboring *Salmonella* strains in patients with typhoid fever, and most reports are related to the prevalence of these strains in food products and animal specimens[23,24]. Hence, this study investigated the antibiotic resistance patterns and ESBLs prevalence in clinical isolates of *S. Typhi* from Lagos, Nigeria.

The majority of *S. Typhi* isolates (92.6%) were resistant to trimethoprim/sulfamethoxazole, while all of them were susceptible to imipenem and nitrofurantoin. In addition, more than 50.0% of isolates were resistant to third-generation cephalosporins. In line with the current results, Saeed et al.[7], from Pakistan, reported resistance rates of 0.0%, 96.3%, and more than 50.0% against carbapenems, trimethoprim-sulfamethoxazole, and third-generation cephalosporins, respectively. Likewise, in another study by Adamu et al.[25] from Nigeria, all *Salmonella* species isolated from typhoid patients were susceptible to imipenem. Differences in the source of isolates studied, the methods used to evaluate the antibiotic susceptibility, the presence or absence of antibiotic consumption surveillance programs, use of antibiotics in the food production and veterinary industries, and the types and races of the population studied may be the reasons for these contradictory results. In contrast to the current study, El-Tayeb et al.[26], from Saudi Arabia, revealed a high resistance rate of *S. Typhi* isolates against nitrofurantoin. As a result of nitrofurans being used as a feed supplement or treatment by veterinary professionals, particularly those working in the poultry industry, *Salmonella* has exhibited high resistance to this antibiotic[26].

In this study, the resistance rates against amoxicillin-clavulanate, gentamicin, and ciprofloxacin were 18.5%, 22.2%, and 33.3%, respectively. Umair and Siddiqui[27] from Pakistan (62.7%) and Katiyar et al.,[28] from India (96.9%), reported higher resistance rates for ciprofloxacin compared to the current research. However, Katiyar et al.[28] from India and Maharjan et al.[29] from Nepal showed lower resistance rates for aminopenicillins compared to this study. It should be noted that plasmidic resistance to quinolones may be undetected by disk diffusion methods leading to treatment failures. It is recommended that the microdilution test for ciprofloxacin be performed prior to the assumption of quinolone susceptibility. In this study, the rate of MDR *S. Typhi* isolates (55.6%) was higher than the previous studies by Umair et al. (44.6%)[27] and Shah et al. (20.0%)[30] from Pakistan and Maharjan et al. (0.0%)[29] from Nepal.

The results of antibiotic resistance for *Salmonella* isolates are different in various countries, even in the diverse regions of the same country. There are many possible explanations for these discrepancies, including differences in the source of isolates studied, the methods used to evaluate the antibiotic susceptibility, the presence or absence of antibiotic consumption surveillance programs, use of antibiotics in the food production and veterinary industries, and the types and races of the population studied. The findings of this study showed that the third-generation cephalosporins and trimethoprim-sulfamethoxazole are unsuitable choices for empirical therapy of typhoid fever. Furthermore, due to the high and increasing drug resistance in the world, it is recommended that imipenem and quinolones be used for the treatment of infections caused by MDR or extensively

drug-resistant Salmonella species and not for routine empirical therapy.

So far, the ESBL-producing *S. Typhi* isolates are rarely reported in clinical samples from African countries, including Nigeria[20] and Democratic Republic of the Congo[31]. However, in Asian countries, including Pakistan[7], Bangladesh[32], Kuwait and United Arab Emirates[10], Philippines[11], and Sri Lanka[33], there have been several reports of ESBL-harboring *S. Typhi*. The presence and emergence of ESBL genes may be due to the exchange of plasmids IncA, IncY and IncX3 carrying these factors between gram-negative bacteria[7]. Furthermore, the occurrence of ESBL-harboring *S. Typhi* isolates has been reported from food and animal origin[13,14,24].

In this study, the CDT showed that 64.7% (n = 11/17) of third-generation cephalosporin-resistant isolates were ESBL producers, while the results of M-PCR revealed that all of them harbored ESBL genes. In total, the prevalence of ESBL-positive isolates detected by phenotypic and genotypic methods were 40.7% (n = 11/27) and 63.0% (n = 17/27), respectively. These discordant CDT and PCR test results may be due to the presence of plasmidic AmpC enzymes. A major detection challenge of ESBLs is AmpC-producing isolate, since ESBL inhibitors like clavulanic acid do not inhibit AmpC enzymes, which makes it difficult to identify ESBL production. As a result, such microorganisms are incorrectly classified as non-ESBL-producing[34]. The bla_{TEM} (70.6%) was the most prevalent ESBL, followed by bla_{CTX-M} (58.8%) and bla_{SHV} (29.4%). In line with the current study, Saeed et al.[7] from Pakistan, reported bla_{TEM} (51.0%) as the most frequent gene followed by bla_{CTX-M} (42.6%). However, contrary to our study, bla_{SHV} was not detected in their study. In another study by Phoba et al.[31] from Democratic Republic of the Congo, a typhoid fever case of *S. Typhi* harboring TEM-1 and CTX-M-15 has been reported using whole genome sequencing. Furthermore, Ahamed Riyaaz et al.[33] from Sri Lanka, reported an *S. Typhi* isolate that harbored CTX-M, TEM, and SHV β-lactamases. This study had some limitations. During this study, a small population of patients was enrolled. As a result, our findings may not represent the entire country. The effects of various socioeconomic factors, antibiotic prescriptions regimen, and use of over-the-counter (OTC) drugs on the resistance profile of isolates were not investigated. The ESBL genes were not sequenced. To explain the other possible mechanisms of antibiotic resistance among *S. Typhi* isolates, further molecular studies covering other resistance genes are suggested.

In conclusion our study revealed the high-frequency rate of MDR *S. Typhi* isolates harboring ESBL genes in patients with typhoid fever from Lagos, Nigeria. These isolates showed high resistance rates against first-line antibiotics for typhoid fever treatment. Therefore, to deal with this severe emergence of MDR *S. Typhi* in the country, diagnostic facilities and rational prescription and use of antibiotics and proper infection control are recommended measures.

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