

Antibiotic Resistance in Extended Spectrum Beta-Lactamases (Esbls) *Salmonella* Species Isolated from Patients with Diarrhoea in Calabar, Nigeria

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Abstract

Objective: This study seeks to evaluate the prevalence of *Salmonella* producing ESBLs strains by phenotypic methods and their profile of drug resistance amongst patients with diarrhoea due to gastroenteritis and enteric fever in Calabar, Nigeria.

Methods: Stool samples were collected from 256 patients in Calabar with diarrhoea due to enteric fever and gastroenteritis. They were examined for *Salmonella* infection. Isolation and detection of *Salmonella* species was done following the standard ISO 6579:2002/Amd2007 method. Modified double disc synergy and phenotypic confirmatory tests were used to determine ESBL-producing *Salmonella* species.

Results: *Salmonella* isolates were recovered from 44 (17.2%) stool samples; 24 (9.4%) were ESBLs producers and 20 (7.8%) were non-ESBLs producers. Eight (33.3%) and 3 (15.0%) strains of both ESBL producing and non-ESBL producing respectively, demonstrated resistance against 7 of the 8 antibiotics used in this study. Resistance against 3rd generation cephalosporin was observed in 34 (77.3%) of the *Salmonella* strains against ceftazidime, 26 (59.1%) against cefotaxime while all 44 (100%) *Salmonella* strains were resistant against ceftriaxone.

Conclusion: The presence of ESBL *Salmonella* amongst isolated strains should not be overlooked. We recommend continuous surveillance of antimicrobial resistant strains and the rational use antimicrobials agents.

Keywords: *Salmonella*; Antibiotic susceptibility; Multidrug resistance; ESBL; Double disk synergy; Calabar-Nigeria

Abbreviations:

ESBL: Extended Spectrum Beta Lactamase; UCTH: University of Calabar Teaching Hospital; GTH: General Hospital Calabar; NNH: Nigerian Navy Hospital Calabar

Introduction

The emergence and spread of antibiotic resistant strains particularly the detection of the Extended Spectrum Beta-lactamases (ESBLs) *Salmonella* species, is fast becoming an emerging world threat [1]. ESBLs frequently occur in *K. pneumoniae* but recent studies have increasingly reported its occurrence in the *Salmonella* species and other gram-negative organisms [2,3]. Such *Salmonella* strains pose significant health problems worldwide by virtue of their acquisition of resistance against most beta-lactam antibiotics, which were previously used for treatment of *Salmonella* infections.

ESBLs are enzymes capable of hydrolyzing penicillin, cephalosporin and oxymino- β -lactam compounds (i.e. cefuroxime, third generation cephalosporin and aztreonam) with cephamycins and carbapenems as exceptions. Most ESBLs belong to the Ambler class "A" β -lactamases and are inhibited by β -lactamase inhibitors (clavulanate, sulbactam and tazobactam) [1]. ESBLs are plasmid-mediated and as a

result, are easily transmitted among members of the Enterobacteriaceae family [2].

This potential exacerbates the spread of resistance against β lactams and other commonly used antibiotics including quinolones and aminoglycosides [4]. This has further limited the therapeutic options available and hence complicated the treatment and management of infections by such organism.

The determination of antibiotic susceptibility pattern and multidrug resistant pattern of infectious organisms is therefore necessary to provide a vivid guide for physicians to make informed drug choices during the management and treatment of patients. It is also important in ensuring the effective and quick treatment of *Salmonella* infections as well as other infections without aggravating the illness. This minimizes antibiotic resistance and cost of treatment [5].

However, there is a dearth of data on not only the *Salmonella* producing ESBL species but also their multi drug resistance pattern amongst human subjects. The aim of this study was therefore to evaluate the prevalence of *Salmonella* producing ESBLs strains by phenotypic methods and their profile of drug resistance amongst patients with diarrhoea due to gastroenteritis and enteric fever in Calabar, Nigeria.

Methods

This study was carried out within the Calabar Municipal Local Government Area of Cross River State, Nigeria. Calabar has an area of 142 km² and a population of 179,392 by the 2006 census. The average annual rainfall is about 3070 mm with concentration between March and November while the dry season lasts from November to March. Calabar municipality is surrounded by large expanse of water bodies, yet faced with the problem of potable and good quality water. Most of the inhabitants of Calabar are traders [6].

Study design

This was a descriptive study, designed to investigate the presence of ESBLs among *Salmonella* strains isolated from patients attending three major hospitals within Calabar municipal. The sample size used in this study was calculated based on the formula by WHO using an expected prevalence of 16% with an absolute precision of 0.05 [7,8].

Sample collection

Stool samples were collected from 256 diarrhoeal patients between December 2014 and May 2015. Out of which, 42 attended the Calabar General Hospital while 80 and 134 patients were from the University of Calabar Teaching Hospital, and the Nigerian Navy Hospital, Calabar respectively. Faecal samples were collected from patients prior to any antibiotic therapy. Samples were collected in labeled sterile universal containers containing glycerol saline and were transported to the Laboratory for bacteriological analysis within 4 hours [9].

Ethical consideration

The protocol for this study was approved by the Ethical Committees of the selected hospitals in this study. Approval was also obtained from the Cross River State Ministry of Health, conveyed via CRS/MH/CGSE-H/018/Vol/123 and the Health Research Ethical Committee of University of Calabar. Consent was sort from all study participants and guardians for those bellow 16 years of age prior to the collection of samples.

Isolation and identification of *Salmonella* species

The isolation and detection of *Salmonella* from the experimental samples was carried out based on the traditional ISO 6579:2002/Amd:2007 method (ISO, 2002). In the pre-enrichment stage, a 1 in 10 dilution of the stool sample was made in 10% modified buffered peptone water to promote the growth of all members of Enterobacteriaceae and to recover injured cells. This was carefully mixed and incubated at 35°C for 24-48 hours. Secondly, 0.1 ml of the pre-enrichment homogenate was transferred into 10 ml of Modified Semi-solid Rappaport Vassiliadis (a selective enrichment medium for *Salmonella*). Following incubation at 40-41°C for 24 hours, streaking on *Salmonella*-Shigella (SS) agar, MacConkey's agar (MAC) and Xylose Lysine Deoxycholate Agar (XLDA) selectively plated a loop-full. This was incubated for 18-24 hours at 35-37°C. All the bacteriological media as well as reagents and some materials required were obtained from Hardy Diagnostics (HD) and BD Difco.

Confirmation of *Salmonella* isolates

Prior to the further confirmation of presumptive *Salmonella* isolates, typical *Salmonella* colonies on selective solid plates were sub-

cultured on nutrient agar (NA) to obtain pure and distinct colonies and the isolated *Salmonella* strains were confirmed by various biochemical reactions [10]. The colonies were then picked by means of a sterile needle, stabbed and streaked on pre-prepared Triple Sugar Iron Agar (TSIA) and Christensen agar (CA) slants. Alkaline slope/acid butt, with or without the production of H₂S and gas, on TSIA slant and Urease negative on Christensen agar slant were suggestive of *Salmonella*. Further confirmation of *Salmonella* species was conducted based on the standard biochemical techniques in order to identify the isolates, which belonged to the genus *Salmonella*. This involved the use of Lysine Decarboxylation (LCD) test, β-galactosidase test, Acetone production test and Indole production test. Confirmation was further supplemented by using commercially available polyvalent *Salmonella* antisera kit specific for all group and type-factor *Salmonella* antigens. Here, a loop full from *Salmonella* isolates that satisfy all the confirmation procedures were then emulsified with a drop of normal saline (0.85% NaCl) on a microscopic glass slide [11]. The preparation was gently stirred and observed for auto-agglutination. If there was no auto-agglutination, a drop of the polyvalent antisera was added and gently agitated by rocking back and forth and observed for agglutination. Those that showed agglutination were considered to belong to the genus *Salmonella*. Those isolates that showed auto-agglutination were not suitable for *Salmonella* confirmation.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion method based on the Clinical and Laboratory Standards Institute (CLSI) in Mueller-Hinton agar plates with commercially available antibiotic discs (Hardy Disc-HDx) [12]. The confirmed *Salmonella* isolates were cultured on pre-prepared Brain Heart Infusion (BHI) agar (HARDY Diagnostics) plates and incubated at 37°C for 24 hours in to obtain confluent growth for sensitivity test. A loop-full of isolates from BHI plates was dispensed in sterile normal saline to match the 0.5 McFarland Turbidity Standard (1.0 × 10⁸ CFU/μl) for sensitivity tests as described elsewhere [13]. 100 μl of the bacterial suspension was then inoculated on the iso-sensitivity test agar plates, and the excess was siphoned by means of sterile Pasteur pipettes. The plates were allowed to dry at room temperature in a laminar flow. The pre-determined antibiotic disks were then dispensed into the bacterial lawn by means of a sterile pair of forceps and gently pressed to ensure complete contact with the agar. The discs were positioned 15 mm way from the edge of the plate and 25 mm away from each other. The plates were incubated at 35-37°C for 18 to 24 hrs. The diameters of the zones of inhibition were read and interpreted in accordance with standards approved by the Clinical and laboratory standard institute [14]. The antibiotic discs used included: chloramphenicol (30 μg), sparfloxacin (5 μg), amoxicillin (30 μg), gentamicin (10 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ceftazidime (30 μg) and amoxicillin/clavulanic acid (25/5 μg).

Screening for ESBLs producing *Salmonella* strains

Salmonella isolates that showed resistance against third generation cephalosporin by disc diffusion method were selected for further detection of ESBL production by double disc synergy test (DDST) [15]. A disc of amoxicillin-clavulanic acid (30 μg) was placed on the centre of the Muller-Hinton agar (HardyDiagnostics) plate, which was previously inoculated with resistant strain. Each cephalosporin disc of ceftriaxone-30 μg, cefotaxime-30 μg and ceftazidime-30 μg was placed around the amoxicillin-clavulanic acid disc 20 mm apart and incubated

for 18-24 hours at 37°C. A clear extension of the edge of the inhibition zone of any of the antibiotics towards the central disc (amoxicillin-clavulanic acid) disc was interpreted as positive for ESBL production [16]. *K. pneumoniae* 700603 was used as a control strain for a positive ESBLs production while *E. coli* 25922 was used as a negative control for the ESBLs production.

Phenotypic disc diffusion method for ESBLs confirmation

Confirmation of ESBL phenotype was done by phenotypic confirmatory disc diffusion method, using both ceftazidime alone and in combination with amoxicillin-clavulanic acid. The ESBL positive *Salmonella* strains as determined by double disk synergy test were used for confirmation. The *Salmonella* strains were confirmed as ESBLs producers if they showed a ≥ 5 mm increase in the zone diameter of inhibition when ceftazidime was used in combination with clavulanic acid compared to the zone diameter of inhibition when ceftazidime was used alone. Screening for ESBL was considered positive if the zone diameter of inhibition for ceftazidime, cefotaxime and ceftriaxone were respectively ≤ 27 mm, ≤ 22 mm and ≤ 25 mm [17].

Salmonella strains that showed resistance against more than three different groups of antimicrobials such as fluoroquinolones, aminoglycosides, cephalosporin, were considered multi drug resistant strains [18].

Availability of data

The data supporting our findings cannot be share now because it is a product of an on-going study.

Statistical analysis

The data obtained in this study were analysed by means of the IBM SPSS Statistics 20.0. The difference in drug resistance between ESBL and non-ESBL producing *Salmonella* strains was evaluated by means of the Chi-square test. P-value less than 0.05 was considered statistically significant.

Results

Stool samples were obtained from 256 patients presenting with diarrhoea due to enteric Fever and gastroenteritis. These patients attended 3 major hospitals in Calabar. In total, *Salmonella* was recovered from 44 patients (17.2%) of the 256 sampled. A breakdown of the isolates from the patients by hospital attended is shown in Table 1. The percentage number of *Salmonella* isolated from all 3 hospitals showed that the University of Calabar Teaching Hospital (UCTH) isolated the most strains from the samples. The Nigerian Navy Hospital Calabar (NNHC) follows this while General Hospital Calabar (GHC) isolated the least. There was significant difference in the isolation rate of *Salmonella* species in the three hospitals in Calabar ($\chi^2=9.890$, $df=2$, $p=0.007$) (Table 1).

Hospitals	No. Of samples Collected	No. (%) Of Confirmed <i>Salmonella</i> species
UCTH	42	14(33.3)
GHC	80	9(11.3)
NNHC	134	21(15.7)
Total	256	44(17.2)

$$\chi^2 = 9.890, df = 2, p=0.007$$

Table 1: Distribution of *Salmonella* species by the three hospitals in Calabar.

Of the 256 stool samples obtained, 184 (71.9%) did not reveal *Salmonella* species and 28 (10.9%) were not suitable for the *Salmonella* confirmation, therefore, 212 (82.8%) were considered negative for *Salmonella*. However, 44 (17.2%) were confirmed positive for *Salmonella*, out of which 24 (9.4%) demonstrated ESBLs production based on the double disc synergy test while 20 (7.8%) did not (Figure 1). All 24 presumptive ESBLs producing *Salmonella* isolates were confirmed to be ESBL producers as revealed by the phenotypic confirmatory test.

The result of multidrug resistance was recorded for both groups of ESBLs and non-ESBLs producers. The drug resistance of *Salmonella* isolates to antimicrobial agents commonly used in the treatment of diarrhoea is shown in Table 2. All the isolates demonstrated resistance against ceftriaxone.

Antibiotics	ESBL producing <i>Salmonella</i> spp. (n=24)	Non-ESBL producing <i>Salmonella</i> spp. (n=20)	Total No. (%) (N = 44)	P-value
Chloramphenicol (30 µg)	18 (75.0)	5 (25.0)	23(52.3)	0.001*
Sparfloxacin (5 µg)	16 (66.7)	3 (15.0)	19(43.2)	0.001*
Amoxicillin (30 µg)	20 (83.3)	7 (35.0)	27(61.4)	0.001*
Gentamicin (10 µg)	8 (33.3)	11 (55.0)	19(43.2)	0.127
Ceftriaxone (30 µg)	24 (100.0)	20 (100.0)	44(100)	NA
Cefotaxime (30 µg)	19 (79.1)	7 (35.0)	26 (59.1)	0.004*
Ceftazidime (30 µg)	24 (100.0)	10 (50)	34 (77.3)	0.000*
Amoxicillin/Clavulanate (25/5 µg)	0 (0)	0 (0)	0 (0)	NA

* - Significant difference; NA – Not Applicable

Table 2: Pattern of antimicrobial resistance between ESBL and non-ESBL producing *salmonella* species.

The *Salmonella* isolates (both ESBLs and non-ESBLs producers) were resistant against ceftriaxone while all *Salmonella* strains were susceptible to amoxicillin/clavulanic acid. Resistance against 3rd generation cephalosporin was observed in 34 (77.3%) patients for ceftazidime in 34 (77.3%), 26 (59.1%) for cefotaxime and 44 (100%) for ceftriaxone in all *Salmonella* strains.

Amongst the 24 ESBL producers, 6 (25.0%) strains were sensitive to chloramphenicol, while 16 (66.7%) were resistant to sparfloxacin. Amongst non-ESBL producing *Salmonella* isolates, resistance was observed against cefotaxime and ceftazidime in 7 (35.0%) and 10 (50%) strains, respectively. Also, Amoxicillin resistance was seen in 20 (83.3%) and 7 (35.0%) strains of ESBL and non-ESBL producers respectively, while gentamicin resistance was observed in 8 (33.3%) and 11 (55.0%) strains of ESBL and non-ESBL producers respectively.

Among the *Salmonella* strains, ESBL producers demonstrated significantly more antimicrobial resistance against all the antibiotics used in this study (with the exception of gentamicin) compared to non-ESBL producers.

Among the 24 ESBL producers, 8 (33.3%) *Salmonella* strains exhibited resistance against 7 of the 8 antibiotics used in this study whereas only 3 (15.0%) *Salmonella* strains demonstrated resistance to 7 out of the 8 antibiotics used among the 20 non-ESBL producers.

Discussion

Non-typhoidal salmonellosis poses a potential threat to human health and still persists as an important enteric infection in humans, particularly in the young, the elderly and the immunocompromised [19]. Although the disease is most of the time self-limiting and confined within the bounds of the intestinal tract, in some cases the disease has been known to exhibit dangerous consequences when it spreads beyond the intestines or when the immune system of the host is compromised in one way or the other [5].

The injudicious and indiscriminate use of antibiotics particularly in the developing countries has led to an increasing incidence of

antibiotic resistance among *Salmonella* strains, worldwide [16]. Recently, ESBLs have emerged as an important driver of drug resistance.

In spite of the widespread distribution of ESBLs, the prevalence and phenotypic characteristic among clinical isolates may differ with different geographical locations [2]. Prevalence of ESBLs between *E. coli* and *Klebsiella* has been frequently demonstrated in several countries, but it is worth recognising the emergence of ESBLs in *Salmonella*, which now confers serious clinical problem [4,20].

This study revealed a prevalence of 17.2% *Salmonella* species isolated from stool samples from patients presenting with gastroenteritis in Calabar (Figure 1). This is relatively low when compared with the results of other studies such as the 24.56% recorded in west Bengal, India, the 32.1% recorded in North Karnataka, India, the 53% in Mumbai and the 62.3% recorded in Pondicherry [21-23]. However, a recent study in Bangladesh reported that the distribution of *Salmonella* species shows distinct seasonality, with higher prevalence occurring during the raining season (May to October) [3]. Therefore, the relatively low prevalence of *Salmonella* as observed in this study could account for the fact that the period of collection of samples fell within the dry season (December 2014 to May 2015).

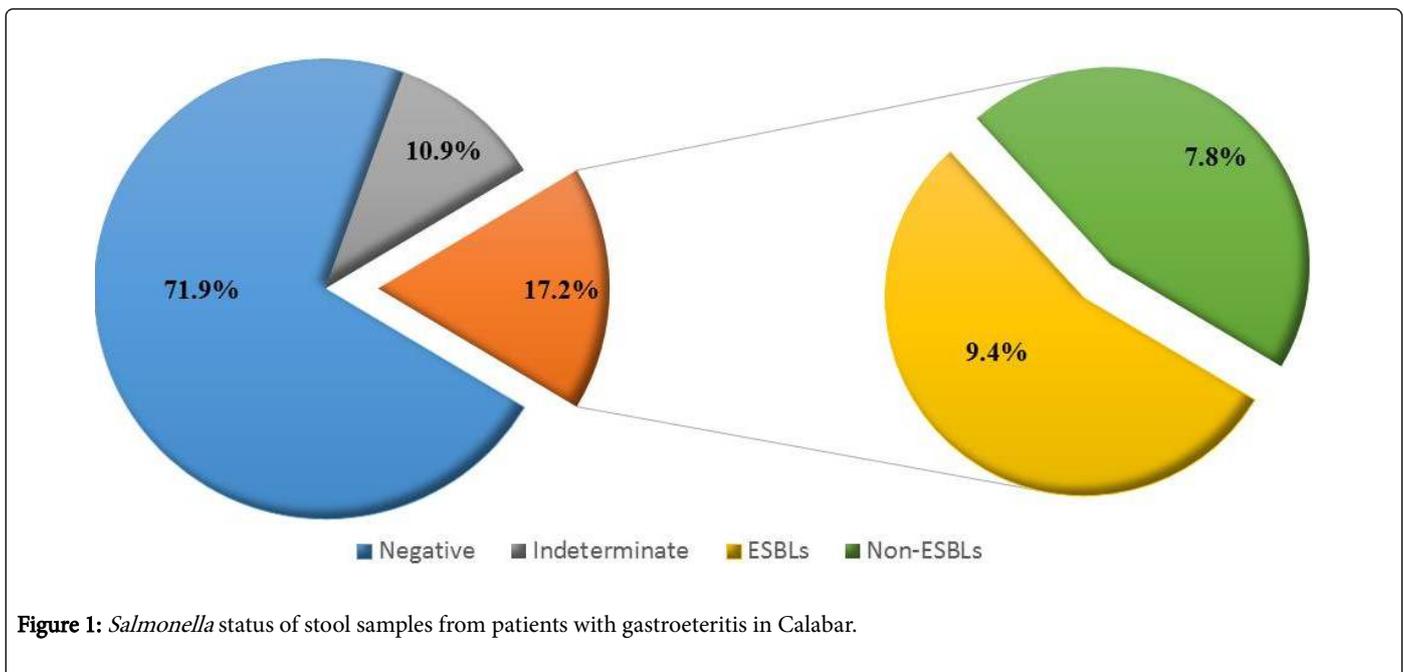


Figure 1: *Salmonella* status of stool samples from patients with gastroenteritis in Calabar.

This study also demonstrated a 9.4% prevalence rate of ESBLs producing *Salmonella* strains by using the phenotypic confirmatory test method (Figure 1). A limited number of studies have been carried out to determine the prevalence of ESBLs producing *Salmonella* strains with zero to very low prevalence such as in the study carried out in Abuja, Nigeria [24]. However, the prevalence of ESBLs producing *Salmonella* species was lower in this study compared to other species of the Enterobacteriaceae family [4,25,26].

Furthermore, antimicrobial resistance was significantly higher among ESBL producers than non-ESBL producers (Table 2). Ceftazidime and ceftriaxone resistance in this study were found to be particularly related to ESBLs production in *Salmonella* strains. This corroborates the recent finding that indicates that most detected ESBLs have special affinity to degrade ceftazidime [2]. Another report also

shows ceftazidime to be efficient in screening isolates as potential ESBLs producers [20].

Also, resistance due to ESBLs was demonstrated by using the phenotypic confirmatory disc diffusion method, which is relatively cheaper and easy to carry out. However, the sensitivity of this method is inferior when considering Epsilometer test strip (an exponential gradient method of determination of antimicrobial resistance) and molecular detection techniques, which are more sensitive and reproducible for confirmation of false positive results. Nonetheless, they are very expensive and were not used in this study.

The increasing antibiotic resistance among ESBL producing *Salmonella* species is a major public health concern worldwide therefore, surveillance of antimicrobial resistant strains and rational

use of antimicrobial agents is necessary for effective treatment and prediction of occurrence of resistant populations.

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None

Competing Interests

The authors declare that they have no competing interests

Author's Contributions

OJO, BEB initiated planned and completed the study and wrote the initial manuscript. NYY, AOE and UMF analysed data and also reviewed the manuscript. All authors read and approved the final manuscript.

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