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# Incidence of Extended-spectrum beta-lactamase producing *Citrobacter* from Cloacal swabs of apparently healthy turtles at the bank of River Niger in Lokoja, Kogi State, Nigeria

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***Abstract***
*Bacteria-encoded extended spectrum beta-lactamases (ESBLs) are of grave clinical concern to public health globally`. ESBLs-producing bacteria hydrolyze a broader spectrum of beta-lactam antimicrobials compared to their simple parent beta-lactamases from which they are derived. The present study investigated the occurrence of extended-spectrum beta-lactamase producing strains among eleven (11) Citrobacter isolates from the cloacal swabs of apparently healthy turtles at the bank of river Niger in Lokoja, Kogi State, Nigeria. Citrobacter species were earlier identified on the basis of morphological and biochemical characteristics. Double disk diffusion method was employed to detect ESBL production phenotypically while Polymerase Chain Reaction (PCR) analysis facilitated the identification of the prevalence of the genes (blaTEM, blaSHV, blaCTX-M, and blaOXA), which are known to be responsible for ESBL production. Out of the eleven (11) Citrobacter isolates tested, only 2 representing 18.2 % produced ESBL phenotypically. The predominant ESBL-producing genes detected were blaTem (100%), blaOXA (100%) and blaCtxm 8 (72%). Conclusively, the detection of these ESBL-related genes in the two isolates suggests that turtles in the study location harbor citrobacter species that produce enzymes associated with multi-drug resistance. Consequently, zoonotic infections by these strains of citrobacter could pose a challenge to effective treatment with antibiotics available for routine use.*

***Keywords:*** *ESBLs, Antibiotic resistant, Beta-lactamase, Phenotypic, Isolates, Citrobater*

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## I. Introduction

Beta-lactam antibiotics are a broad group of molecules that are naturally produced by different organisms: molds belonging to *Penicillium* spp. and *Cephalosporium* spp. for penicillins and cephalosporins, respectively, and bacteria belonging to different species for monobactams and carbapenems (Margherita *et al*., 2021). These antimicrobials share the same mechanisms of action and a similar structure with penicillin, having specific signature of the presence of β-lactam ring (Pancu *et al*., 2021). The antibiotics (β-Lactam) are regarded as the most widely prescribed antibacterial drugs due to their low toxicity and broad spectrum activity (Margherita *et al*., 2021). Antibacterial action requires the binding to penicillin binding proteins (Nauta *et al*., 2021), preventing them from closing the vulnerable ends on dividing bacteria and causing the natural intra-bacterial hyperosmotic pressure to rupture the bacteria, in a bactericidal effect.

The action of β-Lactam antibiotics is counteracted by different resistance mechanisms including the reduction of membrane porins, improvement in the number of efflux pumps, alteration of penicillin-binding proteins and hyper-expression of extended spectrum β-Lactamases (Fernández and Hancock, 2012; Moyá *et al.*, 2012; Uddin *et al*., 2021). Among them, the most common strategy is the expression of β-Lactamase, enzymes that hydrolyze the amide bond present in all β-lactam compounds (Palacios *et al*., 2020). β-Lactamase enzymes are produced by an increasing number of clinically relevant (both Gram-positive and Gram-negative) bacteria, as defensive strategy against β-lactam antibiotics (Alfei and Zuccari, 2022). The enzymes are disseminated across opportunistic pathogens such as Enterobacteriaceae (e.g. *Escherichia coli*) and non-fermenting organisms

(e.g. *Pseudomonas aeruginosa*) (De Rosa *et al*., 2019). Infections due to extended-spectrum beta-lactamase (ESBL)-and carbapenemase (CPM)-producing *Enterobacteriaceae* family of bacteria impose a major global issue because they are usually resistant to multiple antimicrobial agents (Worku, 2022). β-Lactamase enzymes are known to be inhibited by clavulanate, sulbactam and tazobactam; unfortunately, the activity of these inhibitors is limited to β-lactamase enzymes of class A, not including carbapenemases, and is weak against class C but absent against carbapenemases of group B and D (Olsen, 2015). ESBLs-production potentiates multi-drug resistance to antimicrobials and exacerbates serious infections by Enterobacteriaceae (Worku, 2022); there is a clear indication to screen *Citrobacter* isolates from the study location for the possibility of ESBLs-production.

The present study was therefore designed to investigate *Citrobacter* isolates from the cloacal samples of apparently healthy turtles at the bank of River Niger in Lokoja, Nigeria for ESBLs-production using a standard phenotypic assay (Double disk diffusion method) and a quantitative polymerase chain reaction (PCR) was employed to determine ESBLs-related gene (blaTEM, blaSHV, blaCTX-M, and blaOXA) expressions.

## II. Materials And Methods

***Equipment and materials***

**Equipment:** Autoclave (LDZX-30FBS, England), Incubator (Genlab, UK), Oven (Gulfex Medical and Scientific, England), Refrigerator (Haier Thermocool, China), Weighing balance, Hot plate, Bunsen burner, and Laminar flow hood were employed in the study.

**Materials:** Test tubes, Beakers, Conical flasks, Inoculating loop, Sterile needles and syringes, McCartney bottles, Hand gloves, non-absorbent cotton wool, Aluminum foil, Alcohol (95%), Potassium hypochlorite solution, Paper tape, Petri dishes, Universal sterile bottles, Sterile distilled water, and Mueller Hinton agar (Oxoid, UK) were used in the study

***Study Area***

The sampling was carried out at the bank of River Niger in Lokoja, Kogi State, Nigeria. Kogi State is found at the confluence of the Rivers Niger and Benue being one of the 36 States in the federation. Lokoja lies between Latitude $7^0\,45^1\,27.56^0 - 7^0\,51^1\,04.34^{11}$N and Longitude $6^0\,41^1\,55.64^{11} - 6^0\,45^1 36.58^{11}$.

***Bacterial isolates and identification of Citrobacter species***

A total of 245 samples of cloacal swabs collected from apparently healthy turtles at the bank of River Niger in Lokoja, Kogi State, Nigeria were used in the studies. The isolates were subjected to standard confirmatory tests, which included Gram staining, growth on Sulfide-Indole-Motility (SIM), Simon citrate, Methyl Red- Voges Proskauer (MR-VP), Triple Sugar Iron (TSI) agar, urea agar, malonate, Blood agar, MacConkey agar, Salmonella shigella agar, Eosin methylene blue agar (MacFaddin, 1999) and Microbat 24 E identification tests.

***ESBL Detection***

ESBL production was detected by using the double disk diffusion method. In keeping with the Clinical and Laboratory Standards Institute (CLSI) recommended guidelines, ESBL screening was performed by means of disk diffusion using cefpodoxime (10 µg) and ceftazidime (30 µg) disks. The ESBL phenotype was confirmed by means of the double disk diffusion method, using antibiotic disks containing a combination of cephalosporin plus clavulanic acid (cefpodoxime (10 µg) and ceftazidime (30 µg) plus amoxicillin-clavulanic acid (20:10 µg). The tests were interpreted in accordance with the CLSI guidelines. Regardless of the zone diameters, an increase in zone diameter ≥ 5 mm for an antimicrobial agent tested in combination with clavulanic acid, in comparism with its zone size when tested alone, indicated probable ESBL production (CLSI, 2014). Tests for each strain were done in triplicate and results were represented as the mean of three replicates of the zone of inhibition diameter obtained.

***Genotypic confirmation of Antibiotic Resistant gene using PCR***

Polymerase chain reaction amplification of blaTEM, blaSHV, blaCTX-M, and blaOXA were conducted with a thermal cycler (Garrec *et al*., 2011) using the following primers:

| | | |
|---|---|---|
| Bla TEM: TEM-F | $5^1$-GTA TCC GCT CAT GAG ACA ATA ACC CTG-$3^1$ | |
| TEM-R | $5^1$-CCA ATG CTT AAT CAG TGA GGC ACC-$3^1$ | 918bp |
| bla SHV: SHV-F | $5^1$ -CGC CTG TGT ATT ATC TCC CTG TTA GCC -$3^1$ | |
| blaSHV-R | $5^1$-TTG CCA GTG CTC GAT CAG CG- $3^1$ | 842bp |
| blaCTX-M-F | $5^1$ -CGC TTT GCG ATG TGC AG - $3^1$ | |
| blaCTX-M-R | $5^1$ -ACC GCG ATA TCG TTG GT- $3^1$ | 550bp |
| blaOXA –F | $5^1$ - ATGCGTGTATTAGCCTTATCG- $3^1$ | |
| blaOXA-R | $5^1$ -CATCCTTAACCACGCCCAAATC- $3^1$ | 265bp |

All the ESBL genes were amplified under the following conditions:
Initial denaturation at 95 $^0$C for 5 minutes, followed by 35 cycles of denaturation at 95 $^0$C for 30 seconds, annealing at 55 $^0$C for 30 seconds, and 72 $^0$C for 1 minute, with a final extension at 72 $^0$C for 5 minutes. The amplicons were run on 1% agarose gel. The gels were stained with ethidium bromide, and bands observed at the desired position were photographed using an ultraviolet light transilluminator.

## III. Results

The production of ESBL was performed by double disc synergy test. About 2 (18.2%) out of the 11 isolates produced Beta-lactamase phenotypically, while 9 (81.8%) were non–Beta Lactamase producers (Plate 1; Figure 1).

**Plate 1:** Phenotypic detection of Beta-lactamase production in *Citrobacter* species isolated from the cloacal swabs of apparently healthy Turtles in Lokoja, Nigeria

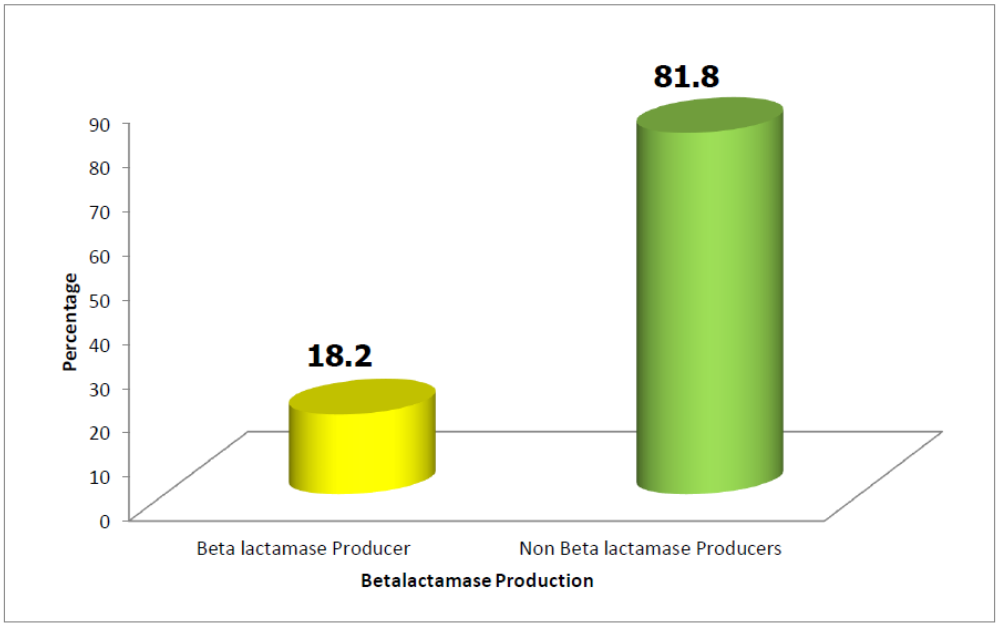


**Figure 1:** Phenotypic detection of Beta-lactamase production in *Citrobacter* species isolated from the Cloacal swabs of apparently healthy Turtles in Lokoja, Nigeria

***Polymerase Chain Reaction Results***

Figure 2 shows the amplification of *blaTEM*, *blaOXA*, *blaSHV*, and *bla*CTX-M genes of the 11 isolates of *Citrobacter* species from turtles on electrophoretic gel with their respective amplicon lengths: 918 bp, 265 bp, 842 bp, and 550 bp respectively. The result shows that all the isolates contained *blaTEM* 11(100%) and *blaOXA* 11 (100%), but 8 (72%) isolates contained *bla*CTX-M and 1 (9.1%) contained *blaSHV*. The isolates were: Lane1(L1)= 1B, L2= 2B, L3= 58B, L4= 67B, L5= C6, L6= C9, L7= C17, L8= D2a, L9= D9a, L10= D16a, and L11= D9b. M represented the molecular weight which ranged from 100 to 1000 base pairs.

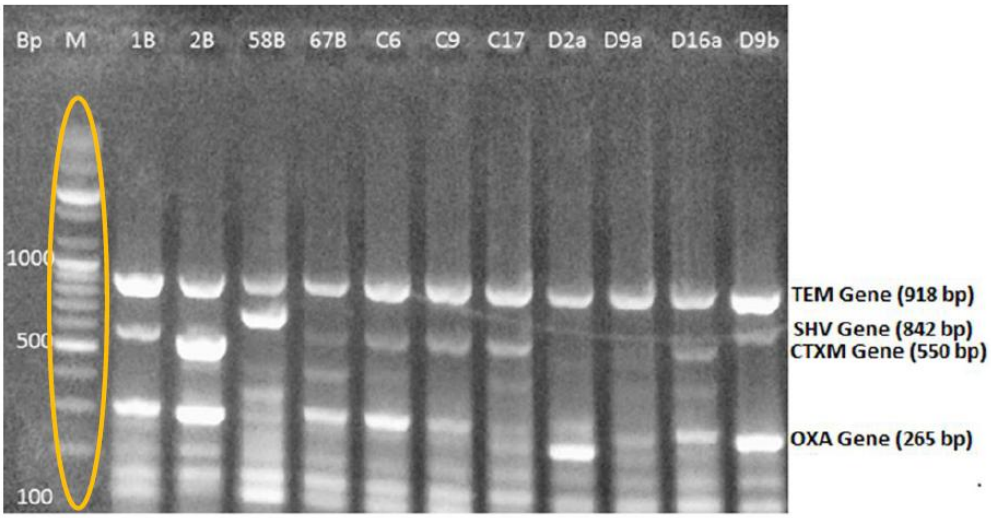


**Fig. 2:** Amplification of the ESBL genes of the *Citrobacter* isolates from the Cloacal swabs of apparently healthy Turtles in Lokoja by Gel electrophoresis. *Tem* (918 bp), *Oxa* (265 bp), Shv (842 bp) & *C*tx-M (550 bp).

## IV. Discussion

ESBLs are enzymes that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and the monobactam (aztreonam). Infections with ESBL-producing organisms have been associated with poor outcomes (Ben-Ami *et al.*, 2009). Evaluation for the presence of ESBL was carried out using double disc diffusion method. The method indicated the presence of ESBL in 2 (18.2%) of the 11 multidrug resistant *Citrobacter* isolates that were found to be resistant to at least four (4) and more groups of antibiotics. The ESBL producing *Citrobacter* observed in this study was comparable with the report of Kanamori *et al*. (2011) who reported 19.3% ESBL producing *Citrobacter* species isolates from the hospital environment in Japan. However, this was far` lower than the findings of Rizvi *et al.* (2010) and Uma *et al.* (2004) who had reported 62% and 86.50% respectively amongst the hospital isolates (in North India). Further investigations revealed that the isolates displayed resistance to multiple classes of antibiotics. However, the resistance shown by these *Citrobacter* isolates as exemplified in the low ESBL production observed in this study might be due to other mechanisms of resistance apart from β-lactamase production. This implies that additional resistance mechanisms may have existed but not expressed at the phenotypic level, suggesting a wider spectrum of resistance mechanisms. Xiong *et al.* (2002) observed that ESBL producing speci es were also resistant to other commonly used antibiotics, such as streptomycin, kanamycin, tetracycline, cefepi me,
amoxicillin and imipenem thereby corroborating with the findings in this study. ESBL production confers multi drug resistance which narrows treatment options with consequent health complications particularly with nosocomial infections.

Molecular characterization of ESBL genes in Multi-Drug Resistant (MDR) *Citrobacter* isolates from cloacal swabs of turtles in Lokoja, Nigeria, showed that *blaTEM* 11 (100%), *blaOXA* 11 (100%) and *bla*CTX-M 8(72%) were the predominant ESBL genes relative to *blaSHV* 1 (9.1%) (Figure 2). Yadav *et al*. (2015) observed that bla(TEM) is however the preponderant gene found in *Klebsiella*, *Citrobacter*, *Enterobacter*, and *E. coli* species. These findings are in tandem with the reports of Lei *et al.* (2013) who reported that *bla*CTX-M is the most widely distributed gene encoding extended-spectrum β-lactamases in humans globally. Shahi *et al.* (2013) also reported 12 (75%) ESBL production among *E. coli* strains from diabetic ulcer, in which *bla*CTX-M were the most prevalent [10 (62.5%)] strains. The *blaTEM* and *blaOXA* were detected in 9 (56.3%) of the strains, while *blaSHV* was present in 8 (50%). The occurrence of CTX-M enzymes in pathogenic bacterial isolates poses serious problems to control of the infection caused by them in a community setting; routinely prescribed antibiotics may not be effective in the treatment (Woodford *et al*., 2004). CTX-M genes is recognized in various

disease conditions of epidemic proportion caused by multiple-antibiotic-resistant bacterial organisms worldwide (Naseer *et al*., 2006; Seyedjavadi *et al.,* 2016).The detection of CTX-M resistant genes among *Citrobacter* isolates from the study area therefore emphasizes the epidemic potential of this organism.

## V. Conclusion

The results of the study showed that 2 (18.2 %) out of the 11 *Citrobacter* isolates from the cloacal swabs of apparently healthy turtles at the bank of River Niger in Lokoja, Kogi State,Nigeria produced ESBLs phenotypically. The genes predominantly responsible for ESBLs production were identified as *blaTem* 11 (100%), *blaOxa* 11 (100%) and *blaCtxm* 8 (72%).

**CONFLICT OF INTEREST**

All authors hereby declare that there was no conflict of interest.

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