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Original article

Isolation and molecular identification of extended spectrum beta-lactamase producing enterobacteria from clinical samples in Kogi State, Nigeria

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ABSTRACT

Extended-spectrum beta-lactamases (ESBLs) are a rapidly evolving group of β-lactamases that could hydrolyze third generation cephalosporins and aztreonam but are inhibited by clavulanic acid. Bacteria strains that can produce ESBLs have become a serious global challenge due to their increasing resistance to a broad array of antibiotics. Infections due to ESBL producers range from uncomplicated urinary tract infections to life threatening sepsis. Clinical samples (stool, urine, sputum and wound swabs) were collected from children and adults from four different hospitals in Kogi State. The samples were examined for Enterobacteria using standard bacteriological techniques. The physiological and biochemical characteristics of recovered isolates were compared with those of known taxa. The phenotypic screening and PCR analysis of ESBL producing bacterial strains were carried out according to standard protocol. The prevalence of Enterobacteria was 314 (39.25) with the age group 31-40 recording the highest prevalence 78(24.84%). *E. coli* was found to be the most frequently occurring Enterobacteria 165(52.55), other Enterobacteria isolated were *Klebsiella* species 55 (17.55), *Salmonella* species 34 (10.83), *Providencia stuartii* 15 (4.78), *Alcaligenes faecalis* 16 (5.10), *Proteus* species 10 (3.18), *Enterobacter* species 10 (3.18), *Citrobacter* species 2 (0.64), and *Morganella morganii* 7 (2.23). Out of 314 Enterobacteria isolates, 43(13.69) were ESBL positive. This study provides evidence of the dissemination of ESBL and a need for appropriate monitoring of the prescription and use/misuse of antibiotics to reduce the prevalence of Extended Spectrum Beta-Lactamases.

Keywords: Extended-spectrum beta-lactamases, Antibiotics, Enterobacteria, Clinical samples, Kogi State.

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INTRODUCTION

The beta-Lactamases are a group of bacterial enzymes that hydrolyzes Beta-lactam antibiotics. The first Beta-lactamases discovered are the broad-spectrum TEM-1, TEM-2, and SHV-1. The product of mutation of the genes that encode these beta-lactamase enzymes gave rise to the current extended-spectrum *β*-lactamases (ESBLs). These ESBL enzymes were initially recognized in clinical isolates in the 1980s; they were derived from the TEM or SHV types of β-lactamases, by point mutation in the parent enzymes which did not possess extended-spectrum β-lactam substrate activity. More than 200 ESBLs have been identified excluding the TEM, SHV and CTX-M types, other clinically relevant types of ESBLs include the VEB, PER, GES, TLA, IBC, SFO-1, BES-1 and BEL-1 types [1].

The Extended-Spectrum *β*-lactamases are chromosomal or plasmid-mediated and occur because of a spontaneous mutation that takes place in the active site of the wild-type beta-lactamase enzyme by adding 4-6 new amino acids, thereby extending their hydrolytic properties [2].

The Enterobacteriaceae are Gram negative, facultative anaerobes, and non-sporing bacilli. These bacteria have become one of the most important causes of nosocomial and community acquired infections. They could cause urinary tract infection, respiratory tract infection, blood stream and wound infections. Increasing rates of antimicrobial resistance have become a worldwide problem predominantly caused by Gram negative bacteria [3].

The extended-spectrum *β*-lactamase-producing enterobacteriaceae have been isolated from clinical settings in different parts of the world, and as plasmid-mediated enzymes, they can hydrolyze penicillins, cephalosporins and monobactams, but not cephamycins and carbapenems. They are inhibited by classical and newly developed β-lactamase inhibitors such as clavulanic acid, sulbactam, tazobactam, avibactam, or nacubactam, among others [4].

These ESBL-producing bacteria may also be multiple resistant to other classes of antimicrobial agents such as aminoglycosides, trimethoprim/sulfamethoxazole, and quinolones. These Multi drug-resistant (MDR) strains cause infections which are difficult to treat. The World Health Organization (WHO) has declared infections caused by MDR bacteria as an emerging global health problem of major public health concern [1].

MATERIALS AND METHODS

Study Area

The study was carried out in Kogi State, which was carved out of the former Kwara and Benue States in 1991. Kogi State lies on latitude $7^0$49N and longitude $6^0$44E at an altitude of 45-125m. The state is divided into 3 senatorial districts- Kogi Central, Kogi East and Kogi West.

Study Population

The study was carried out on 800 patients comprising of children and adults of both genders attending, Federal University Teaching Hospital, Lokoja; Prince Abubakar Audu University Teaching Hospital, Ayingba; General Hospital,

Kabba; and Diagnostic and Reference Hospital, Okene, Kogi State. The sample size of 800 was determined using Cochran equation [7] and the prevalence rate of 12.5% [Duru *et al*. 2000] was used. The inclusion criteria were inpatient diagnosed with clinical infection, while patients who were unwilling to fill the questionnaire or give permission for sample collection and also those who were on antibiotics were excluded in the study.

Collection of samples

Mid-stream urine samples were collected in sterile universal bottles, stool samples were collected from different patients in sterile universal containers, sputum samples were also collected in sterile containers, and wound swabs were collected aseptically from patients using sterile swab sticks. All sample bottles, containers, and swabs were tightly sealed and well labelled. The samples were stored at low temperature after collection and transported to the laboratory of the Federal University Teaching Hospital Lokoja, for analysis.

Isolation and identification of isolates

All culture media were prepared according to the manufacturer's specification. Samples were inoculated onto Nutrient agar, MacConkey agar, Deoxycholate citrate agar, Eosin methylene blue agar, and Cysteine lactose and electrolyte deficient agar.

Urine samples were inoculated unto the prepared media using wire loop and incubated at 37 °C for 24 hours. Stool samples were also picked using wire loop and inoculated on the prepared media and incubated at 37 °C for 24 hours. A loop of sputum was inoculated unto the prepared media and incubated at 37 °C for 24 hours. The wound swabs were inoculated on the agar surface and streaked, plates were incubated at 37 °C for 24 hours. The isolates were examined for their various colonial morphology.

Gram staining

A drop of distilled water was placed at the center of a clean grease free glass slide. A sterile wire loop was used to pick a colony from the culture, a smear was made on a clean grease free glass slide and allowed to air dry. The air-dried smear was heat fixed by passing it over a flame. The fixed smear was stained with crystal violet for 60 seconds, washed off with clean water. Lugol's iodine was added for 60 seconds and then washed off with clean water. The smear was decolourized with 95% ethyl alcohol and washed off with clean water, after which the counter stain safranin was added for 60 seconds and wash off with clean water. The slide was air dried and examined microscopically using 100X oil immersion objective lens [8].

Biochemical tests

The following biochemical tests were conducted; Citrate utilization test, Methyl red test, Oxidase test (Cytochrome oxidase), Triple Sugar Iron (TSI) agar test, Indole test, and Catalase test, for identification and characterization.

Antibiotics susceptibility testing

Screening of the isolates against the following antibiotics was performed using the modified Kirby Bauer disc diffusion method to determine the antibiotics susceptibility pattern of Enterobacteria isolates as recommended by EUCAST guidelines [9].

Discrete colonies of the organism to be tested were picked with a sterile wire loop and emulsified in 5 ml of sterile nutrient broth. The broth culture was incubated for 6 hours at 37 ^{o}C to achieve the turbidity of the 0.5 McFarland standard. The turbidity of the suspension was matched to the turbidity standard. Müeller-Hinton agar plates were inoculated with the test organism using a sterile swab stick. The swab was evenly streaked over the surface of the medium, rotating the plate to ensure even distribution. With the petri dish lid in place, it was allowed for 15 minutes for the surface of the agar to dry. Antibiotic discs (Amikacin 10µg, Amoxicillin-Clavulanic acid 20/10µg, Cefotaxime 30µg, Ceftazidime 30µg, Ceftriaxone 30µg, Cefoxitin 30µg, Cefuroxime 30µg, Cefatrizine 30µg, Cefalexin 30µg, Ceftiaxone 30µg, Sulphamethoxazole/Trimethoprim 25µg, Tetracycline 30µg) were evenly distributed on the inoculated plate using sterile forceps. The plates were inverted and incubated at 37 ^{o}C for 24 hours. After incubation, the plates were observed for zone of inhibition. The diameter of each zone of inhibition was measured in millimeter and interpreted accordingly [8].

Phenotypic ESBL Detection

The presence of ESBL-producing isolates were phenotypically detected by the Double Disk Synergy Test (DDST) [10,11,12]. In a double disc synergy test, synergy was determined between a disc of amoxicillin+clavulanic acid placed centrally on the plate, with Ceftazidime, Cefotaxime and Ceftriaxone placed at a distance of 20 mm apart from the center disc on the surface of the culture plate. The test organism with a zone of inhibition around the test antibiotic disc greater than 5mm towards amoxycillin/clavulanic disc was considered to produce ESBL [13].

Polymerase Chain Reaction (PCR)

PCR analysis for the detection of Extended Spectrum beta lactamase genes was carried out on all phenotypically confirmed Extended Spectrum beta lactamase producing Enterobacteria.

16S rRNA Amplification

The 16s rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95 ºC for 5 minutes; denaturation, 95 ºC for 30 seconds; annealing, 52 ºC for 30 seconds; extension, 72 ºC for 30 seconds for 35 cycles and final extension, 72 ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

RESULTS AND DISCUSSION

Prevalence of Enterobacteria in clinical samples

The prevalence of Enterobacteria in clinical samples from Kogi State was determined. From the total of 800 clinical samples collected the overall prevalence of *Enterobacteria* was 314 (39.25). This indicated a moderately high presence of *Enterobacteria* within the study

population. [14] noted *Enterobacteria* species as well-known representative bacteria causing nosocomial infections as such, they are commonly isolated in clinical specimens. Higher *Enterobacteria* prevalence was reported in a study by [15], who reported a prevalence of 42.7% in blood, urine, sputum, wound, and stool samples from 384 hospitalized patients at Debre Berhan Comprehensive Specialized Hospital in Ethiopia. [16] also reported 78.7% *Enterobacteria* prevalence from a total of 650 clinical specimens in Nepal. [17] also recorded a higher prevalence of 57.02% from a total of 228 samples collected from selected hospitals in Abuja, Nigeria. The lower prevalence of Enterobacteria observed in the present study can be attributed to differences in the study population, seasonal and geographic location.

Across age groups, the highest prevalence was observed in the age group 31-40 with a prevalence of 24.84%. This is similar to a study by [18] where the age group 31-40 year recorded the highest prevalence of Enterobacteria (*Escherichia coli* and *Klebsiella* species) in urine samples in Benin, Nigeria. The high prevalence of Enterobacteria recorded in the above age groups suggests that individuals within this age range may be more susceptible to Enterobacteria infections compared to other age groups. This could be attributed to biological or behavioural factors such as immune system function, lifestyle behaviours, and exposure to environmental risk factors associated with certain professions or activities in middle-aged adults.

Gender differences in prevalence of Enterobacteria were minimal within each age group and statistically non-significant. Males 161 (51.27) recorded higher prevalence of *Enterobacteria* compared to females 153 (48.73). The observed results indicate that gender may not play a significant role in influencing the prevalence of Enterobacteria infections among the studied population. Variation in prevalence between the male and female gender can be attributed to physiological and anatomical differences in both sexes [19,20].

Table 1: Prevalence of Enterobacteria from Clinical Samples

Age Range (years)	No. of Samples Examined	No. of Positive Samples (%)	Gender	
			Positive Male Samples (%)	Positive Female Samples (%)
11-20	131	44(14.01)	23(52.27)	21(47.73)
21-30	222	70(22.29)	34(48.57)	36(51.43)
31-40	201	78(24.84)	41(52.56)	37(47.44)
41-50	117	58(18.47)	28(48.28)	30(51.72)
51-60	70	31(9.87)	16(51.61)	15(48.39)
≥61	59	33(10.51)	19(57.58)	14(42.42)
Total	**800**	**314(39.25)**	**161(51.27)**	**153(48.73)**

Frequency of occurrence of Enterobacteria isolated from clinical samples

On the frequency of Enterobacteria isolated from clinical samples, the most common Enterobacteria isolated was *Escherichia coli* 165(52.55), other Enterobacteria isolated include *Klebsiella* species (17.55), *Salmonella* species (10.83), *Alcaligenes faecalis* (5.10), *Providencia* species (4.78), *Proteus* species (3.18), *Enterobacter* species (3.18), *Morganella morganii* (2.23), and *Citrobacter* species (0.64).

Similar findings were reported by Agegnehu *et al.* (2020), who recorded *Escherichia coli* (44.4%), *Klebsiella pneumoniae* (27.8%), *Klebsiella oxytoca* (8.33%), *Providencia* spp. (5.6%), *Citrobacter diversus* (4.16%),

Enterobacter cloacae (2.8%), *Proteus mirabilis* (2.8%), and *Klebsiella ozaenae* (4.16%) as the most common Enterobacteria isolates. [17] observed a similar trend in their study on the prevalence of Enterobacteriaceae among children in selected hospitals in Abuja, Nigeria, recording *Escherichia coli* (47.69%) as the most dominant species, followed by *Salmonella* (24.62%), *Klebsiella* (15.38%), *Proteus* (8.46%), *Shigella* (1.53%), and *Citrobacter* (2.3%).

These Enterobacteria species are well-established as significant clinical pathogens, frequently causing nosocomial infections that result in substantial morbidity and mortality [21,22, 23], as such the need for continued surveillance and effective infection control measures.

According to gender some Enterobacteria species were more prevalent in females, including *Escherichia coli* (52.12%), *Klebsiella* species (54.55%), *Proteus* species (60.0%), *Enterobacter* species (70.0%), and *Citrobacter* species (100.0%). In contrast, *Salmonella* species (67.65%), *Providencia stuartii* (60.0%), *Alcaligenes faecalis* (81.25%), and *Morganella morganii* (71.43%) were more common in males. The results showed a gender-based variation in the prevalence and distribution of specific Enterobacteria species. These variations can be attributed to differences in biological, behavioural, and social factors [24] Physiological and anatomical differences in sexes have been noted as an important factor in the susceptibility of various diseases and conditions, including Enterobacteria-related infections. Differences including hormonal variations, urinary tract anatomy and function, immune system responses and metabolic rates, determine susceptibility to infections. In addition to physiological and biological factors, behavioural factors such as hygiene practices, healthcare-seeking behaviour and occupational exposure also determine susceptibility to infections [25].

Table 2: Frequency of Occurrence of Enterobacteria Isolated from Clinical Samples

Isolate	Total Number of Isolates (%)	Gender		X^2	p-value
		Male	Female		
Escherichia coli	165(52.55)	79(47.88)	86(52.12)	0.16	0.69
Klebsiella species	55(17.55)	25(45.45)	30(54.55)	1.00	0.32
Salmonella species	34(10.83)	23(67.65)	11(32.35)	12.96	0.00
Providencia species	15(4.78)	9(60.00)	6(40.00)	4.00	0.05
Alcaligenes faecalis	16(5.10)	13(81.25)	3(18.75)	38.44	0.00
Proteus species	10(3.18)	4(40.00)	6(60.00)	4.00	0.05
Enterobacter species	10(3.18)	3(30.00)	7(70.00)	16.00	0.00
Citrobacter species	2(0.64)	0(0.00)	2(100.00)	100.00	0.00
Morganella morganii	7(2.23)	5(71.43)	2(28.57)	17.64	0.00

Phenotypic detection of ESBL isolates

In this study, Extended-spectrum beta-lactamases (ESBLs) production was identified in 13.69% of the isolated Enterobacteria species. This prevalence is comparatively lower than those reported by [26], [27] and [28]. [26] documented a 62.2% ESBL prevalence among *Enterobacteriaceae* isolated in Addis Ababa, Ethiopia, while [27] recorded a 61.5% ESBL prevalence rate among *Enterobacteriaceae* from selected tertiary hospitals in South-Eastern Nigeria. Additionally, [28] documented a prevalence of 28.9% among *Enterobacteriaceae* isolated from clinical samples in Ilorin Metropolis, Kwara State, Nigeria.

The lower ESBL prevalence rate observed in the present study compared to these other studies may be attributed to factors such as differences in geographical locations, study populations, sample sizes, and laboratory methods used for ESBL detection [29]. Additionally, variations in antibiotic usage practices, infection control measures, and healthcare settings among different regions and populations could also contribute to the observed differences in ESBL prevalence rates [28].

Among the screened *Enterobacteriaceae* species *Escherichia coli* 16 (37.21%) and *Klebsiella* species 16 (37.21%) were the most prevalent ESBL-producing isolates, this is similar to the report by [28], who

recorded *Escherichia coli* (17.3%) as the major ESBL producer followed by *Klebsiella pneumoniae* (9.3%). The high occurrence rate of ESBL in *E. coli* and *Klebsiella* species in the present study raises significant public health concerns. This is particularly worrisome considering that these members of the *Enterobacteriaceae* family, are widely recognized as the predominant causative agents of both nosocomial and community-acquired infections [30,31].

The production of extended-spectrum beta-lactamases (ESBLs) in *Enterobacteriaceae* isolates can be attributed to various genetic mechanisms, including the presence of ESBL-encoding genes such as CTX, SHV, and TEM. ESBLs are frequently encoded by plasmids that carry genes conferring resistance to beta-lactam antibiotics. These genes have the potential to be horizontally transferred between bacteria, thereby facilitating the dissemination of ESBL-producing strains [32].

Table 3: Phenotypic Detection of ESBL Positive Isolates in the Study Population

Organisms	No. of Isolates	No. of ESBL Positive Isolates (%)	No. of ESBL Negative Isolates (%)
Escherichia coli	165(52.55)	16(37.21)	149(54.98)
Klebsiella species	55(17.52)	16(37.21)	39(14.39)
Enterobacter species	10(3.18)	0(0)	10(3.69)
Salmonella species	34(10.83)	0(0)	34(12.55)
Providencia stuartii	15(4.78)	1(2.33)	14(5.17)
Alcaligenes faecalis	16(5.10)	1(2.33)	15(5.54)
Proteus species	10(3.18)	0(0)	10(3.69)
Citrobacter species	2(0.64)	2(4.65)	0(0)
Morganella morganii	7(2.23)	7(16.28)	0(0)
Total	**314**	**43(13.69)**	**271(86.31)**

Detection of 16S rRNA gene in selected bacteria isolates

Plate I shows the amplified 16S rRNA of selected bacteria isolates using the agarose gel electrophoresis. Lanes 1-9 represent the bacteria (1=*Klebsiella variicola*, 2=*Escherichia coli*, 3=*E. coli*, 4=*Citrobacter freundii*, 5=*Morganella morganii,* 6=*Citrobacter freundii*, 7=*Klebsiella pneumoniae* 8= *Alcaligenes faecalis*, 9=*Providencia stuartii*) tested for 16SrRNA which are represented with gene bands (1500bp). The 16SrRNA is used to identify bacteria present within a given sample to the genus and/or species level.

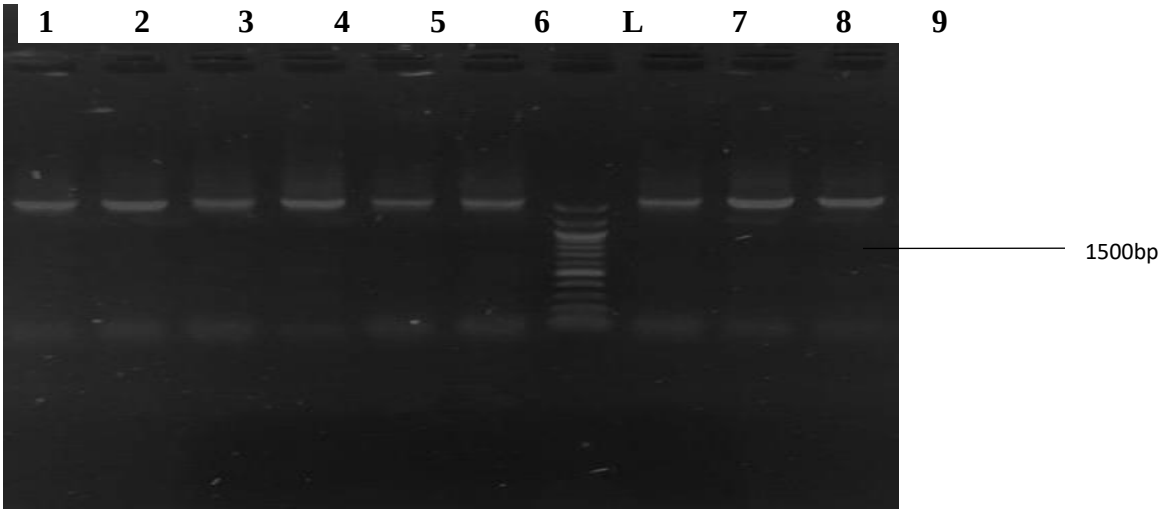

Plate I: Agarose gel electrophoresis showing the amplified 16S rRNA.
Key: Lane 1-9 represents the amplified 16srRNA at 1500bp (1=*Klebsiella variicola*, 2=*Escherichia coli*, 3=*E. coli*, 4=*Citrobacter freundii*, 5=*Morganella morganii,* 6=*Citrobacter freundii*, 7=*Klebsiella pneumoniae* 8= *Alcaligenes faecalis*, 9=*Providencia stuartii*). lane L represents the DNA ladder

DECLARATION

Ethics Approval and Informed Consent

Ethical approval for this study was obtained from the ethical review board of the Kogi State Ministry of Health with reference number MOH/PRS/465/V.1/020. All participants were duly informed of the objectives of the study and the protocol for sample collection. All participants signed an informed consent form. Participation was voluntary.

Authors' Contribution

APO, KFA, ANU and SKD conceptualized the study. APO, KFA, ANU and SKD designed the study. APO participated in fieldwork and data collection. APO, KFA, ANU and SKD performed the data analysis; APO, KFA, ANU and SKD interpreted the data. APO prepared the first draft of the manuscript, reviewed by APO, KFA, ANU and SKD. All authors contributed to the development of the final manuscript and approved its submission.

Disclosure of Conflict of Interest

None

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