Journal of Biosciences and Medicines, 2020, 8, 42-55
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Occurrence of Extended Spectrum Beta Lactamase Encoding Genes among Urinary Pathogenic *Escherichia coli* and *Klebsiella pneumoniae* Isolates Obtained from a Tertiary Hospital in Gombe Nigeria

Adamu Yarima[1*], Ali Ahmed Haroun[2], Timothy Bulus[2,3], Mohammed M. Manga[4]

[1]Department of Plant Science, Bioresource Development Center Michika, National Biotechnology Development Agency, Abuja, Nigeria
[2]Department of Biological Sciences, Nigerian Defence Academy, Kaduna, Nigeria
[3]Department of Biochemistry, Kaduna State University, Kaduna, Nigeria
[4]Department of Medical Microbiology and Immunology, Gombe State University and Federal Teaching Hospital Gombe, Gombe, Nigeria
Email: *adamuyerima08@gmail.com

How to cite this paper: Yarima, A., Haroun, A.A., Bulus, T. and Manga, M.M. (2020) Occurrence of Extended Spectrum Beta Lactamase Encoding Genes among Urinary Pathogenic *Escherichia coli* and *Klebsiella pneumoniae* Isolates Obtained from a Tertiary Hospital in Gombe Nigeria. *Journal of Biosciences and Medicines*, **8**, 42-55.
https://doi.org/10.4236/jbm.2020.89004

Received: June 23, 2020
Accepted: September 6, 2020
Published: September 9, 2020

Abstract

This study was conducted to assess the occurrence and nature of extended-spectrum beta lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from patients who presented with urinary tract infection at Federal Teaching Hospital Gombe. Isolates collected were recovered on MacConkey agar at 35˚C and were identified as members of *Enterobacteriaceae*, and further screened for antimicrobial susceptibility and resistance by disc diffusion method. Isolates resistant to oxyimino-cephalosporins were confirmed as ESBL producers using Double Disks Synergy Test (DDST). The study shows 66% resistance to ceftriaxone (30 µg) in *K. pneumoniae*, which was the highest value recorded and a 51% resistance to cefpodoxime (10 µg) in *E. coli*. The sensitivity of *E. coli* and *K. pneumoniae* isolates to cefpodoxime (10 µg) were 49% and 33.9% respectively. ESBLs were detected among 40% (40/100) of *E. coli* and 54.13% (59/109) of *K. pneumoniae* isolates. Molecular characterization of ESBL encoding genes among *E. coli* isolates using multiplex-PCR showed 10% prevalence of SHV gene and 5% prevalence for CTX-M gene while TEM gene was not detected. In *K. pneumoniae* isolates, 5% prevalence was recorded for each of the three genes screened. The study revealed a co-occurrence of SHV and CTX-M in 75% of the *E. coli* and 70% of the *K. pneumoniae* isolates; the occurrence of all the three genes was seen in 10% and 5% of *K. pneumoniae* and *E. coli* respective-

ly. Multiplex-PCR method provided an efficient and rapid detection of ESBL related genes, hence could be used in epidemiological studies among ESBL isolates. Monitoring dissemination and transmissions of ESBL producers are highly recommended for optimum patient care and preventing the spread of multidrug resistant (MDR) pathogens.

Keywords

ESBL, Double Disk Synergy Test, M-PCR, TEM, SHV, CTX-M Genes, Nigeria

1. Introduction

Beta-lactam antibiotics are a class of broad-spectrum antibiotics, consisting of all antibiotic agents that contain a beta-lactam ring in their molecular structures, and are frequently prescribed antimicrobial agents all over the world in treatment of infections caused by Gram positive and Gram negative bacteria [1]. These agents are widely used antimicrobials for humans as well as animals [2]. Beta-lactam antibiotics are named after their molecular core structure, the beta-lactam ring, which forms the center and active part of the drug [2]. There are six different groups of beta-lactam antibiotics based on their mode of actions: Penicillins, Cephalosporins (1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} generations), Carbapenems, Penems, Monobactams and beta lactamase inhibitors like clavulanic acid and sulbactam [3]. The latter group has no antibacterial activity on its own but inhibits the activity of beta lactamase enzymes and is often given in combination with other beta-lactam antibiotics. Beta lactam antibiotics are known to irreversibly inhibit enzymes involved in the final steps of cell wall synthesis [4]. Beta lactamases are bacterial enzymes that provide multi-resistance to beta lactam antibiotics such as Penicillins, Cephalosporins and Carbapenems, although Carbapenems are relatively resistant to beta lactamase. Beta lactamase enzymes break down the antibiotics structure thereby rendering them ineffective against the bacteria.

As bacteria continue to develop resistance against beta lactam antibiotics such as Penicillins and Cephalosporins newer antibiotics were discovered and used in the treatment. However, the bacteria incessantly evolved and changed the existing beta-lactamase enzymes to break down these new antibiotics. This further class of enzymes that can break down these newer antibiotics was referred to as Extended-Spectrum Beta Lactamases (ESBLs) [5]. Extended-spectrum beta lactamases are plasmid-mediated enzymes which have the ability to hydrolyze and inactivate broad spectrum of beta lactam antibiotics such as penicillins, cephalosporins (3^{rd} generation) and monobactams, but are inhibited by beta lactamase inhibitors like clavulanic acid [6]. The beta lactamase enzymes like TEM-1 were first reported in 1965 [7], SHV-1 in 1972 [8] and CTX-M in 1986 [9]. The spread of beta-lactamases may be chromosomal or plasmid mediated [10].

The occurrence of ESBL among pathogenic bacteria is rising and is associated

with increasing treatment failure, morbidity, and mortality, length of hospital stays and overall cost of patient care [11]. Most ESBL plasmids also carry genes conferring resistance to several non-beta-lactam antibiotics apart from encoding genes conferring resistance to the extended spectrum antibiotics [4] [12]. Presently, more than 400 different ESBLs have been identified, and these are clustered into three major groups: TEM, SHV and CTX-M, with 183, 134 and 103 variants, respectively [13]. Among the mentioned ESBL variants, TEM and SHV were the major types in some countries [14]. *Klebsiella pneumoniae* and *Escherichia coli* remain the major ESBL-producing organisms isolated worldwide, but these enzymes have also been identified in several other members of the *Enterobacteriaceae* family and in certain non-fermentors [15].

Urinary Tract Infection (UTI) is one of the most common infections prevalent among both females and males [16]. It is also a common bacterial infection among infants and young children [17]. It can occur anywhere along the urinary tract and mostly caused by the *Enterobacteriaceae* with *E. coli*, and *Klebsiella species* being most prominent [18] [19]. Annually, over 150 million people are diagnosed with UTIs accounting for about 40% of all infections and making it the second most diagnosed infection worldwide [20] [21]. These infections are treated with a variety of antibiotics including beta lactams, beta lactam/beta lactamase inhibitor combinations, fluoroquinolones and carbapenems [22]. Recent studies revealed that, there is an increase in the antibiotic resistance among the urinary tract pathogens worldwide [22] [23] [24]. This increase in antibiotic resistance is associated with increase in Extended Spectrum Beta Lactamase producing isolates, which are a class of organisms that produce beta lactamase enzymes involved in the hydrolysis of extended spectrum beta lactam antibiotics [6]. Identified risk factors for developing ESBL producers include prolonged hospitalization and indiscriminate use/abuse of antibiotics [25]. In Nigeria, several studies about UTIs have been reported from different parts of the country [24] [26] [27] [28] [29]. Even though several studies have been carried out on the prevalence of ESBL, few have been associated with urinary tract infections, especially in north-eastern Nigeria where the prevalence of urinary tract infection is reported to be as high as 33.34% in Gombe [30]. It is in view of this, that this study was designed to evaluate the prevalence and molecular nature of ESBL in isolates obtained from patients with urinary tract infections in Gombe, Nigeria. This work, to the best of our knowledge is the first report of the molecular characterization of urinary tract pathogenic *E. coli* and *K. pneumonia* from patients who presented at Federal Teaching Hospital, Gombe, North Eastern Nigeria.

The economic and healthcare burden resulting from infections due to multidrug resistant bacteria are estimated to be at least €1.5 billion every year [31]. WHO [32] reported that about 1.8 million children are being killed every year due to increase in resistance among bacteria causing pneumonia. Mortality due to resistant bacterial infections exceeds 25,000 annually [31] in Europe. Production of beta-lactamase enzymes remains the most important contributing factor

to bacterial resistance [33].

2. Materials and Methods

2.1. Study Area

Federal Teaching Hospital (FTH) Gombe is a 450 bedded tertiary healthcare institution that was established in 1996 and is located within Gombe, the capital city of Gombe State Nigeria. It has the full complement of almost all medical/surgical specialties including the clinical microbiology laboratory where cultures and antibiotic susceptibility tests are routinely performed.

2.2. Ethical Approval

This study was approved by the Research and Ethics Committee of the Federal Teaching Hospital Gombe.

2.3. Sample Collection and Identification

Two hundred and nine (209) urinary clinical isolates of *E. coli* (n = 100) and *K. pneumoniae* (n = 109) were taken from the Department of Medical Microbiology to Microbiology Laboratory FTH Gombe from September, 2018 to April, 2019. They were recovered on MacConkey agar plates at 35˚C for 18 hours. Suspected bacterial isolates were identified using biochemical tests as based on established testing methods.

2.4. Phenotypic Detection of ESBL Production

Two tests were done; initial screen test using the indicator cephalosporins (such as ceftazidime) and phenotypic confirmatory test as described by Elsayed [34].

2.5. Screening Test

The screening was done by disc diffusion technique. This involves screening for reduced susceptibility to more than one of the indicator antimicrobials (ceftazidime 30 µg, ceftriaxone 30 µg and cefpodoxime 10 µg). Briefly, A loopful of test isolates was suspended into a normal saline to match 0.5 McFarland turbidity standard and were subsequently swabbed on to a surface of Muller-Hinton agar plates using sterile swab stick. The susceptibility discs of Cefpodoxime (10 µg), Ceftriaxone (30 µg) and Ceftadizime (30 µg) were placed 20 mm apart onto the surface of Muller Hinton agar using sterile forceps, leaving 15 mm away from the edge of the Petri dish. After incubation at 37˚C for 18 hours, inhibition zones were measured to the nearest mm. When a diameter zone of ≤22 mm for ceftazidime, ≤25 mm for ceftriaxone and ≤17 mm for cefpodoxime were recorded, the isolates were reported as suspected ESBL [35]. Suspected ESBL positive isolates were confirmed using Double Disk Synergy Test (DDST). *E. coli* (ATCC-25922) and *K. pneumoniae* (ATCC-700603) were used as reference strains.

2.6. Confirmatory Test Using Double Disk Synergy Test (DDST)

The procedure of Amiri [36] was employed with modification. Briefly, the test organisms were swabbed on to a surface of Mueller-Hinton agar plates with a suspension (adjusted to 0.5 McFarland turbidity standard). A susceptibility disk containing amoxicillin-clavulanate (20/10 µg) was placed in the center of the plate, and disks of Ceftriaxone (30 µg) and Ceftadizime (30 µg) were placed around it at a distance of 15 mm apart. Plates were incubated at 37˚C for 18 hours. An enhanced zone of inhibition as shown in **Figure 1** towards the centrally placed disk was considered positive for ESBL [37]. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as quality control strains [35].

2.7. Molecular Identification of SHV, TEM and CTX-M Bla Genes Using Multiplex PCR

The genomic DNA of forty (40) representative samples was extracted using the commercially available kit (Bioneer, USA) for molecular characterization. The genomic DNA extracts were thereafter stored at −20˚C until required for PCR.

The primers employed in the multiplex PCR were those described by Kaur and Aggarwal [38] for detection of blaTEM, blaSHV and blaCTX-M genes. The sequences of the forward and reverse primers are given in **Table 1**.

Figure 1. Double disk synergy test showing enhanced zone of inhibition toward the amoxicillin + clavulanate disk in the center.

Table 1. Primer sequences used in multiplex polymerase chain reactions.

Target	Primer sequence (5'-3')	Molecular Weight (bp)	Melting Temperature (˚C)
TEM-F TEM-R	GTATCCGCTCATGAGACAATA ACCCTG CCAATGCTTAATCAGTGAGGCACC	918	94
SHV-F SHV-R	CGCCTGTGTATTATCTCCCTGTTAGCC TTGCCAGTGCTCGATCAGCG	842	94
CTX-M-F CTX-M-R	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	94

Sequence obtained from Kaur and Aggarwal [38].

Multiplex-PCR was performed using PTC-100 thermal cycler (USA) to detect beta lactamase genes (blaTEM, blaCTX-M and blaSHV) using PCR conditions as described by [39]. The M-PCR was carried out using 1.5 µl of extracted genomic DNA in a 20 µl PCR reaction mixture consisting of 2.5 µl 10× PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 0.5 µl dNTPs (10 mM), 1.5 µl of each primer, 0.5 µl of Taq DNA polymerase, and 9 µl sterile distilled water. M-PCR was performed under the following conditions: Initial denaturation at 94˚C for 1 minute, denaturation at 94˚C for 30 seconds, annealing at 60˚C for 30 seconds, and extension at 72˚C for 1 minute and a final extension at 72˚C for 6 minutes. PCR products were determined by electrophoresis in a 1.5% (g/v) agarose gel.

2.8. Statistical Analysis

Data obtained were statistically described in forms of frequencies, relative frequencies, figures and tables where appropriate. Differences between proportions were analyzed using ANOVA, in which the P values of the null hypothesis when less than 0.05 were considered significant. All statistical calculations were done using IBM SPSS statistic 23 software.

3. Result

3.1. Antibiotic Susceptibility Pattern of *K. pneumoniae* and *E. coli* Isolates to Common Indicator Cephalosporins

The antibiotic susceptibility pattern of *K. pneumoniae* and *E. coli* isolates is presented in **Table 2**. Results showed that the highest resistance (66%) to Ceftriaxone (30 µg) and 61.5% to Ceftadizime (30 µg) were both observed in *K. pneumoniae*, while the least resistance of 51% to Cefpodoxime (10 µg) was recorded in *E. coli*. The highest sensitivity to Cefpodoxime (10 µg) of 49% followed by Ceftadizime (30 µg) of 48% were both recorded in *E. coli*, while the least sensitivity to Ceftriaxone (30 µg) of 33.9% followed by Ceftadizime (30 µg) of 38.5% occurred both in *K. pneumoniae*.

3.2. Molecular Characterization of ESBL Related Genes

Result of the percentage distribution of genotypes among *E. coli* and *K. pneumoniae* isolated from urinary clinical samples is presented in **Figure 2**. From the 40 randomly chosen representative samples (20 for each isolate) subjected to multiplex-PCR to ascertain the possible gene types (blaCTX-M, blaSHV and blaTEM) responsible for the production of beta-lactamases, test isolates were found to harbour one or more genes. The most prevalent among the detected genes (**Figure 3**) is SHV, followed by CTX-M and TEM with 10%, 5% and 0% prevalence in *E. coli* respectively. TEM gene alone was not detected in *E. coli* but in association with other genes. While in *K. pneumoniae* 5% prevalence recorded for all the three genes. SHV + CTX-M was 75% in *E. coli* and 70% in *K. pneumoniae*, while the combination of SHV + TEM showed 5% in both of the isolates. Co-occurrence of all three genes recorded 5% prevalence in *E. coli* and 10% in *K. pneumoniae*.

Table 2. Antibiotic susceptibility pattern in *E. coli* and *K. pneumoniae* isolates.

Antibiotics (μg)	*K. pneumoniae*		*E. coli*	
	No of resistant isolates (%)	No of sensitive isolates (%)	No. of resistant isolates (%)	No. of sensitive isolates (%)
CAZ (30)	67 (61.5)	42 (38.5)	52 (52)	48 (48)
CRO (30)	72 (66)	37 (33.9)	61 (61)	39 (39)
CPD (10)	63 (57.8)	46 (42.2)	51 (51)	49 (49)

Values before parentheses are number observed while those within are percentages. **Note: CAZ:** Caftadizime, **CRO:** Ceftriaxone, **CPD:** Cefpodoxime.

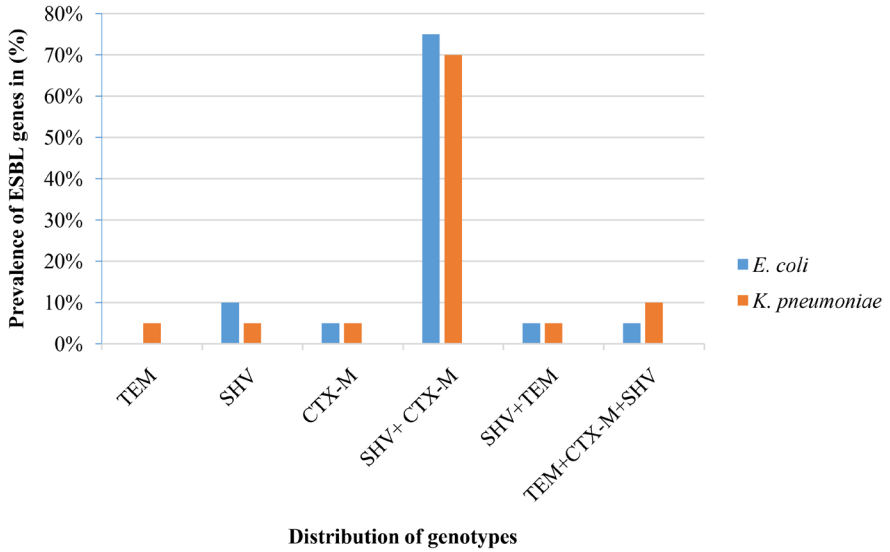

Figure 2. Percentage distribution of genotypes among *E. coli* and *K. pneumoniae* isolated from urinary clinical samples (singly or in combination).

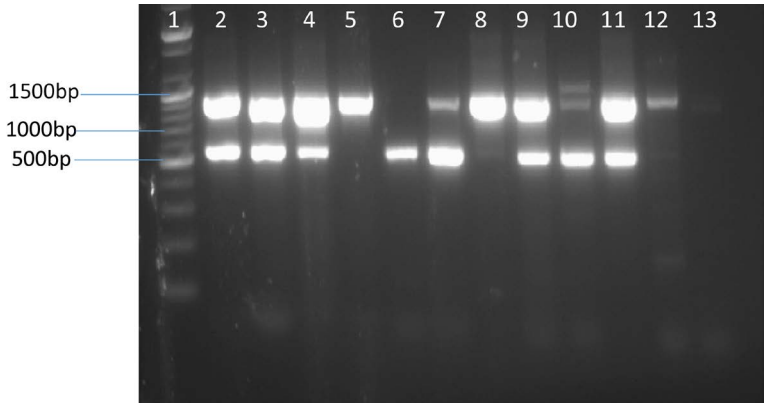

Figure 3. Agarose gel electrophoresis of PCR amplified products showing the presence of CTX-M (550 bp), TEM (918 bp) and SHV (842 bp) genes in *E. coli* isolated from urinary clinical samples. Lane 1 Molecular ladder; Lane 2, 3, 4, 7, 9, and 11; SHV + CTX-M; Lane 5 and 8, SHV; Lane 6, CTX-M; Lane 10, CTX-M + SHV + TEM; Lane 12, (SHV) positive control; Lane 13, Negative control.

4. Discussion

This study was designed to investigate the occurrence, prevalence and molecular nature of ESBL-mediated drug resistance associated with some urinary tract clinical isolates. The resistance of *K. pneumoniae* and *E. coli* isolates to antimicrobial agents tested (ceftriaxone, ceftazidime and cefpodoxime) were observed to be high in this study with the highest resistance recorded in *K. pneumoniae* to ceftriaxone and ceftazidime 66% and 61.5%, respectively. This is consistent with observations made by [40] [41] who reported that *Klebsiella species* were more resistance to third generation cephalosporins compared to *E. coli*. In addition, Chourasia *et al*. [42] buttressed our finding that *Klebsiella species* were the most frequent ESBL producers relative to *E. coli*. In contrast, Farzana *et al*., [43] reported higher ESBL production in *E. coli* over *K. pneumoniae*, *Proteus* spp. and *Pseudomonas spp*. *K. pneumoniae* is known to have some virulence factors like hyperviscosity, polysaccharide capsule, production of endotoxin and carbapenemases and their presence may partly account for the higher occurrence of the resistance seen [44]. The antimicrobial susceptibility pattern observed in this study, in addition to the roles played by the factors mentioned above, may be attributable to the large amount of third generation cephalosporins consumed in our locality as they are relatively cheap and, being an oral antibiotics, easy to administer [18]. Other contributors to this include indiscriminate use/abuse of antibiotics, prolonged hospitalization, incomplete/subtherapeutic antibiotic dose regimens, and the use/misuse of antimicrobials in animal husbandry [25].

The prevalence of Extended Spectrum Beta Lactamase (ESBL) production among *K. pneumoniae* and *E. coli* isolates observed in this study was 54.1% and 40% respectively. These high values were consistent with other reports on ESBL prevalence such as those from India and Egypt [34] [38] where ESBL production as high as 41.5% in *E. coli*, and 54.5% in *K. pneumoniae* were found. Values higher (91% in *E. coli* and 89.2% in *K. pneumoniae*) than observed in this study, have, however, been reported [45] in Aljazira State of Sudan. Much lower results compared to the result from the present finding of 32% in *E. coli*, 20% in *K. pneumoniae*, 20% in *Proteus* spp. and 13% in *Pseudomonas* spp. [43] and 14.29% in *Escherichia coli*, and 7.14% *Klebsiella pneumoniae* in Gombe have also been seen [30].

Recent advances in the field of molecular biology, especially the discovery of Polymerase Chain Reaction (PCR) technique has permitted the targeting of unique genes in microorganisms thereby facilitating their molecular characterization [46] [47]. This approach to identification and detection of disease-causing pathogens is generally adjudged to be very sensitive, fast and accurate [48]. Several PCR types exist with emphasis on specific feature(s)/aspects of the technique, one of which, the multiplex PCR, allows for simultaneous amplification of two or more genes by optimizing conditions that favors annealing of primers to the genes in a single amplification protocol [49]. Therefore, multiplex PCR has had a wide useful application in molecular identification of disease-causing pa-

thogens and, more recently, in the identification of drug resistant gene (s) in diseases associated with organisms such as SHV, CTX-M and TEM genes [38]. In this study, attempt was made to identify, singly or in combination, ESBL related genes using Multiplex-PCR. The study has successfully detected co-occurrence of three major genes related to ESBL in both studied organisms. The occurrence of SHV and CTX-M genes with 75% prevalence in *E. coli* and 70% in *K. pneumoniae*, appear to be the most predominant molecular manifestation of this drug resistance among *K. pneumonia* and *E. coli* isolated from urinary tract-infected individuals. Work by Lal *et al.* [50], reported 67.3% of the two genes occurring together in India and seems to agree with the observation made in this study. According to Zongo *et al.* [51], the coproduction of all the three genes (TEM + SHV + CTX-M) was 10.52% prevalent in the samples they studied in Burkina Faso, and this low figure seems to tally with the 10% and 5% prevalence seen in this work in *K. pneumonia* and *E. coli* respectively. Similar result was also reported by [18] in which all the three genes carried 9.09% prevalence. The most common single gene occurrence observed in this study was with respect to SHV (10% prevalence in *E. coli* and 5% in *K. pneumonia*). Al-Agamy *et al. [52]* similarly observed 6.8% prevalence of SHV in Saudi Arabia. Occurrence of CTX-M gene alone for both isolates was 5% while TEM gene alone was not detected in *E. coli* but had 5% prevalence in *K. pneumoniae*. The co-occurrence of SHV+TEM genes was 5% which is in accordance with the result of [18] 2.27% in Aleppo, Syria. The frequency of occurrence of these beta lactamases encoding genes in both *E. coli* and *K. pneumoniae* were almost the same. The factors that define co-occurrence has not been fully elucidated but could be partly explained by genetic recombination processes in microorganisms.

Significant association between ESBL encoding genes and Urinary Clinical isolates was observed. Higher percentages were reported by [53], in which CTX-M and SHV genes were 28.8% and 13.7% respectively. Much higher prevalence was reported by Ahmed *et al.* [54], in which CTX-M was 71.4% in *E. coli* and 68.4% in *Klebsiella* and TEM was 55.1% in *E. coli* and 58% in *Klebsiella* in Sudan. Yahaya *et al.* [55] reported the prevalence of ESBL genes for *E. coli* and *K. pneumoniae* having 17(38.6%) SHV and 22(66.7%) CTX-M respectively in Borno-North-Eastern Nigeria.

These variations that exist between this study and other findings indicated that the prevalence and type of ESBL and their related genes varied from one geographical region to another [56]. Use and or misuse of antibiotics favour the emergence and spread of drug resistant bacteria globally [32]. This ever-increasing health and economic burden of antimicrobial resistance requires urgent action worldwide to control the situation. Identification of these ESBL-producing isolates and the knowledge of their rates of resistance are of prime importance for the selection of an appropriate antibiotics to be used in the treatment of infectious diseases especially empirically. The predominant factor for escalation of antibiotic resistance is the acquisition of plasmid encoding anti-

biotics resistance genes [53]. Such plasmid can be easily transferred from one organism to another and as such can incorporate genetic material coding for resistance against other antimicrobial classes [18]. Such antibiotic resistance has been reported by Falodun *et al.* [57], to be the outcome of the acquisition of resistance genes through genetic exchange and mutation as well as physiological mechanisms, such as the possession of specific proteins and efflux pump.

5. Conclusion

In conclusion, by this study, we have demonstrated, for the first time, the molecular nature of the occurrence of ESBL among *E. coli* and *K. pneumoniae* isolates causing urinary tract infection in Gombe North-eastern Nigeria. The study showed the co-occurrence of ESBL-related genes in some of the studied isolates and is probably responsible for the observed high resistance to third generation cephalosporins in the locality. To avoid poor identification of antibiotic resistance and the attendant inappropriate antibiotic prescription which may in turn select for new resistance genes, it is recommended that the use of phenotypic tests for ESBL detection be accompanied by the more efficient PCR technique wherever possible. Such molecular methods of detecting ESBL related genes, though sensitive, fast and accurate, are relatively expensive and require specialized equipment and expertise and this should be bored in mind especially when being adopted by countries having many social issues competing for attention from the available limited resource.

Acknowledgements

Authors are thankful to Dr. Yusha'u Muhammad of Microbiology Department, Bayero University Kano for providing the control strains.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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