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Genotypic Analysis of Extended Spectrum B-Lacatamase Producing *Escherichia coli* from Urinary Tract Infections at a Teaching Hospital in Kano, Nigeria

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ABSTRACT

The burden of Urinary Tract Infections has been reported to be on the increase in healthcare settings. *Escherichia coli* has been found to be the most frequently isolated organism. Extended Spectrum Beta Lactamase producing *E. coli* has been shown to be a cause of treatment complication especially in resource limited settings. This study investigated the occurrence and genotypic characteristics of ESBL-producing *E. coli* from urinary tract infections at Aminu Kano Teaching Hospital, Kano, Nigeria. A urine sample size of 500 were collected from inpatients and outpatients with suspected symptoms of UTIs. Identification of bacterial isolates was carried out using morphology and standard biochemical methods. Antibiotic susceptibility testing was performed using the modified Kirby–Bauer disc diffusion method. ESBL production was screened using ceftazidime, ceftriaxone, and cefotaxime, and confirmed by the Double Disc Synergy Test (DDST). Molecular characterization of selected ESBL-producing isolates was carried out by multiplex polymerase chain reaction (PCR) to detect *bla*TEM, *bla*SHV, and *bla*CTX-M genes. Of the 500 samples, 307 (61.4%) yielded no growth, while *E. coli* accounted for 71 (14.2%) of the isolates. Preliminary screening showed 50 (70.4%) potential ESBL producers, of which 20 (40.0%) were confirmed using DDST. All confirmed ESBL producers were found to be resistant to ceftazidime, cefotaxime, ceftriaxone, and amoxicillin-clavulanic acid, while all were susceptible to imipenem. A high level of resistance was observed for gentamicin (95%), ciprofloxacin (95%), cotrimoxazole (95%), chloramphenicol (90%), and nitrofurantoin (65%). Molecular analysis of 20 selected isolates showed *bla*CTX-M was present in 20 (100%) and *bla*TEM in 16 (80%) of the isolates, while *bla*SHV was not detected. Co-occurrence of *bla*CTX-M and *bla*TEM was common. The study demonstrates a considerable burden of ESBL-producing *E. coli* in UTIs, with *bla*CTX-M predominating and frequent co-carriage of *bla*TEM. The high levels of multidrug resistance observed, underscore the need for strengthened antimicrobial stewardship, routine surveillance, and infection control measures to limit the spread of resistant uropathogens in healthcare settings.

Keywords: UTIs, *Escherichia coli*, ESBLs, Imipenem, 3rd generation cephalosporins.

Introduction

Among the infections encountered in healthcare settings, Urinary tract infections (UTIs) have been ranked as very common, and represent an important public health concern, particularly in developing countries. Increasing antimicrobial resistance among uropathogens has further complicated the management of UTIs, with Escherichia coli being frequently implicated among the causative organisms (Akujobi and Ewuru, 2010; Eshetie et al., 2015; Ogefere et al., 2016).

Extended spectrum beta lactamses are produced by organisms that possess enzymes that can inactivate beta lactam drugs. These enzymes have been shown to be destroyed by beta lactamase inhibitors like clavulanic acid. Results from studies carried out in different parts of the world including African countries have shown the presence ESBL producing bacteria (Adeyemo et al., 2020). The growing prevalence of ESBL-producing Enterobacterales is concerning because the genes encoding these enzymes are frequently associated with mobile genetic elements.

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Inappropriate use of antibiotics, including excessive exposure to third-generation cephalosporins, can promote selection favouring the emergence and persistence of resistant organisms. In addition, resistance determinants carried on the same plasmids may confer resistance to antimicrobial classes other than β-lactams, including quinolones, aminoglycosides and sulfonamides (Flores-Mireles et al., 2015). Carbapenems have commonly been considered important treatment strategies for infections resulting from ESBL-producing organisms. However, more frequent administration of these drugs have been associated with emergence of carbapenem-resistant Enterobacteriaceae, thereby further limiting available treatment options (Eshetie et al., 2015; Gajamer et al., 2020; Raja, 2019). Although several investigations of uropathogenic bacteria have focused on phenotypic identification of ESBL production, molecular investigation provides additional information regarding the specific resistance determinants carried by the organisms. Therefore, this work was carried out to establish the occurrence of ESBL-producing E. coli in urinary tract infections and to characterize selected ESBL genes among isolates recovered from patients attending Aminu Kano Teaching Hospital, Kano.

Materials and Methods

Study area

The study was undertaken at Aminu Kano Teaching Hospital (AKTH), a tertiary healthcare facility in Kano State, Nigeria. The hospital was commissioned in 1992 and serves patients from Kano State and surrounding areas. It has approximately 500 beds and comprises several clinical departments supported by consultants, specialists and other healthcare professionals.

Sample size

For population > 10,000, $n = Z^2pq/d^2$

Where n= desired sample size

Z= Standard normal deviate usually set at 1.96

p = proportion in the target population estimated to have a particular characteristic, if no reasonable estimate use 50%

q = 1-p

d= degree of accuracy required usually set at 0.05 level

Prevalence from previous work (Hamza and Kumurya, 2016) from one hospital was reported to be 31.8%

$n = (1.96)^2 \times 0.318 \times (1-0.318)/ 0.05^2$

$n= 3.84 \times 0.318 \times 0.682/0.0025$

$n= 333$

Sample size was calculated as 333 using a prevalence of 31.8% from a study done in one hospital in Kano Metropolis. The sample size was approximated to 500 urine samples

Ethical clearance

Ethical approval for the study was obtained from the hospital's ethics committee (Reference No. NHREC/21/08/2008/AKTH/EC/1742).

Sample collection

The study included both inpatients and outpatients who reported clinical symptoms suggestive of urinary tract infection. Participant authorization was obtained from participating patients, with parental consent obtained for children. Patients included both adults and children. A total of 500 urine specimens were collected. Outpatients were provided with sterile universal containers and instructed to obtain early-morning, midstream clean-catch urine sample. Catheter-derived specimens from appropriate patients and suprapubic urine specimens from infants were collected by nursing staff in the respective wards.

Bacteria identification and Isolation

Urine specimens were inoculated onto CLED agar (Biomark, India) and incubated at 37°C for 24 hours. Specimens with significant bacterial proliferation were further subcultured on MacConkey agar to obtain well-isolated colonies. Gram staining was subsequently performed using standard procedures (Cheesebrough, 2000).

Pure isolates were characterized based on colony morphology and growth characteristics on selective and differential media. Identification was further supported by biochemical testing, including catalase, coagulase, oxidase, Voges–Proskauer, hydrogen sulfide production, urease, methyl red, indole, citrate utilization and sugar fermentation tests, following established procedures (Cheesebrough, 2000; Cowan and Steel, 1993).

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Antibiotic Susceptibility Testing

To determine the susceptibility of the organisms to the antibiotics, the modified Kirby Bauer disc diffusion methods was employed. A standardized bacterial inoculum uniformly inoculated on the surface of Mueller–Hinton agar plates, after which the selected antimicrobial discs were placed on the inoculated surfaces. The inoculated agar plates were maintained at room temperature for approximately 30 minutes to facilitate diffusion before incubation at 37°C for 18 hours. Each isolate was tested in duplicate. Following incubation, inhibition zones were measured in millimetres and recorded (Forbes et al., 2007).

Screening for ESBL Production by Disc Diffusion Test

Resistance to the three antibiotics namely ceftazidime, ceftriaxone and cefotaxime was determined using disc diffusion test as recommended by (Clinical and Laboratory Standards Institute, 2020). A cell suspension that matched the 0.5 McFarland standard was made in nutrient broth using culture of bacterial isolates obtained from overnight cultures on MacConkey agar. A lawn culture was created by spreading the cell solution on Mueller Hinton agar with a sterilised cotton swab. The antibiotic discs were aseptically placed on the petri dishes after the inoculated agar surface had dried. The dishes were then incubated for 24 hours at 37°C. Following incubation, the zones of inhibition's widths were measured in millimetres and noted. Resistance was determined based on the specified interpretive zone diameter criteria for ceftriaxone (30 µg) ≤25 mm, ceftazidime (30 µg) ≤22 mm, and cefotaxime (30 µg) ≤27 mm. In the screening test for potential ESBL development, resistance to at least one antibiotic was regarded as positive (Clinical and Laboratory Standards Institute, 2020).

Confirmation of ESBL production using Double Disc Synergy Test (DDST)

Cefotaxime (30µg), Ceftazidime (30µg), and Amoxycillin-Clavulanic acid (20/10µg) were the three antibiotics used in the Double Disc Synergy Test. Cefotaxime and Ceftazidime discs were placed about 25 mm apart from each side of Amoxycillin-Clavulanic acid on Mueller Hinton Agar.

After a 24-hour incubation period at 37°C, an enhancement in the zone of inhibition towards the clavulanate disc was considered to be suggestive of a possible ESBL producer according to the Clinical and Laboratory Standards Institute (CLSI, 2020).

Molecular Analysis

Molecular characterization of twenty (20) *E. coli* isolates selected on the basis of their being Extended Spectrum Beta Lactamase producers phenotypically was carried out at International Institute of Tropical Agriculture (IITA) Ibadan Oyo state and Inqaba Biotec West Africa. The isolates were analysed for carriage of ESBL genes (Bla_{TEM}, Bla_{SHV}, and Bla_{CTX-M}).

Bacteria culture preparation

The bacteria cultures were prepared using a standard procedure (Dubey, 2009). Luria and Bertani broth media was prepared and sterilized at 121˚C for 15 mins. The Luria and Bertani broth medium was prepared and sterilised for 15 minutes at 121˚C. From isolates on MacConkey plates, single colonies were selected and cultured in 5ml Luria-Bertani (LB) broth medium, and incubated at 37˚C for 18-24 hours. The bacteria culture was collected by centrifuging it for 30 seconds at 25°C in an Eppendorff's tube at 4˚C and 8000 rpm (6800 × g) in a microcentrifuge. After decanting the supernatants, the cells were collected.

DNA Extraction

Extraction of genomic DNA from the *E. coli* isolates was carried out using the ZR Fungal/Bacterial DNA MiniPrep™ Kit (D6005; Zymo Research, USA) in accordance with the manufacturer's recommended protocol.

A 3 mL of overnight bacteria culture grown in Luria–Bertani broth was harvested by centrifuging for 1 min at 10,000xg. The resulting pellet obtained was added to 200 µL of sterile water and subsequently moved to a ZR BashingBead™ lysis tube containing a lysis solution of 750 µL. Centrifugation was carried out for 1 min at 10,000 × g, following clarification, 400 µL of the clear lysate was applied to a Zymo-Spin™ IV spin filter and centrifugation carried out for 1 min at 7,000 rpm.

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A 1,200 µL bacterial binding buffer and filtrate were added to Zymo-Spin™ II-C column. After each step of loading, centrifugation was done for 1 minute at 10,000×g, after which the flow through is disposed. The column was washed with 200 µL of DNA Pre-wash buffer. Afterwards, a 500 µL bacterial DNA wash buffer was added with each washing step preceded by centrifugation.

For DNA recovery, the column was moved into a sterile 1.5mls microcentrifuge with addition of 100µL of elution buffer membrane. After a brief incubation, centrifugation at 10,000xg for 30 seconds, the resulting DNA eluates were collected and stored appropriately until molecular analysis.

Polymerase Chain Reaction (PCR) for detection of Bla_{TEM}, Bla_{SHV}, and Bla_{CTX-M} genes

Polymerase chain reaction (PCR) was performed via thermal cycling conditions recommended by Zymo Research UK, with appropriate optimization of the amplification parameters to enhance product yield and specificity. The required PCR components were combined to prepare the master mix according to the sample size to be analysed. The total master mix volume was computed by multiplying the number of amplification reactions by the total reaction volume of 10 µL. Following thorough homogenization by vortexing, the reaction mixture was distributed into appropriately labelled PCR tubes corresponding to the respective samples. Subsequently, 2 µL of the extracted DNA template was added to each appropriate reaction tube and mixed thoroughly.

The detection of the β-lactamase-encoding genes (TEM, SHV, and CTX-M) was performed using gene-specific primers. The TEM gene was amplified using forward primer 5′-TCGCCGCATACACTATTCTCA GAATG-3′ and reverse primer 5′-ACGCTCACCGG CTCCAGATTTAT-3′, yielding an expected amplicon size of 445 bp. The SHV gene was amplified using forward primer 5′-ATGCGTTATATTCGCCTGTG-3′ and reverse primer 5′-TGCTTTGTTCGGGCCAA-3′, with an expected amplicon size of 747 bp. Similarly, the CTX-M gene was amplified using forward primer 5′-ATGTGCAGYACCAGTAARGTKATGGC-3′ and reverse primer 5′-TGGGTRAARTARGTSACCAG AAYCAGCG-3′, producing an expected amplicon size of 593 bp.

All primer sequences and expected amplicon sizes were adopted from Monstein et al. (2007). PCR amplification started with an initial stage of denaturation at 94°C for 5 min, then a subsequent 35 denaturation cycles at 94°C for 30s, then annealing at 56°C for 30s and then extension for 1 min at 72°C.

The final extension was carried out for 7 mins at 72°C, this was held at 10°C pending further analysis. The products obtained from the PCR amplification were subjected to agarose gel electrophoresis and the bands resulting were viewed in a gel documentation system (Monstein et al., 2007).

Agarose Gel Electrophoresis

Tri-acetic acid – EDTA buffer (TAE) gel was prepared. The number of wells or gene product determined the weight of the agarose and the volume of the TAE. After using thirty wells, 3g of agarose was added to 200 ml of 1TAE and microwaved for 2 minutes to make sure it is completely dissolved.

The mixture was then taken out of the microwave and let to stand for a short while. To stain the DNA products, eight microlitres (8µl) of ethidium bromide (Et Br) were added.

PCR products were separated according to their molecular size by agarose gel electrophoresis. The ethidium bromide-containing agarose solution was dispensed into a casting tray and left undisturbed for approximately 30 min to allow complete gel formation.

After the gel has completely set, the comb and casting components were taken off, and the gel was positioned in the electrophoresis chamber. The chamber was filled with 1× Tris-acetate-EDTA (TAE) running buffer, after which the PCR products were introduced into the designated wells.

A 1-kb DNA ladder (Invitrogen, USA) was included in the first lane to provide a molecular size reference for the amplified fragments. Electrophoresis was conducted for 60 min.

The separated DNA fragments were subsequently examined under ultraviolet illumination, and the resulting banding patterns were recorded using a gel documentation system (Uyanga et al., 2020).

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Results

A total number of 500 urine samples were obtained from patients with suspected case of UTIs, out of which 307 (61.4%) of the samples yielded no growth. *E. coli* was found to make up 71 (14.2%) of the isolates as shown in Table 1.

Table 1: Percentage Distribution of bacterial isolates at the sampling site

Organism	Percentage (%)
E. coli	14.2
K. pneumoniae	13.6
K. oxytoca	0.8
P. mirabilis	3.0
P. aeruginosa	3.2
E. faecalis	1.6
S.saprophyticus	1.2
S. aureus	1.0
No growth	61.4
Total	100

Ceftriaxone, Ceftazidime and Cefotaxime were used to screen for possible ESBL production following the CLSI breakpoint, suspected ESBLs producing *E. coli* were found to be 50 (70.4 %) out of the 71 isolates.

The results for the test to confirm ESBL production using Double Disc Synergy Test showed that out of the 50 potential ESBLs producing *E. coli*, 12 (24.0 %) were ESBLs producers. Using the CLSI (2020) screening criteria using ceftriaxone, ceftazidime, and cefotaxime, 70.4 % of the *E. coli* isolates were classified as potential ESBL producers**,** while the remaining isolates were categorized as not producing ESBLs (Table 2).

Table 2: Percentage distribution of potential ESBLs producing *E. coli* using CLSI breakpoint

Isolates	Number of isolates (%)
Potential ESBLs producers	50 (70.4)
Non ESBLs producers	21 (29.6)
Total	71

Phenotypic testing using the Double-Disk Synergy Test (DDST) revealed that 40.0 % of the *E. coli* isolates were established as ESBL producers (Table 3).

Table 3: Percentage distribution of established ESBLs and Non-ESBLs *E. coli* using Double Disc Synergy Test

Isolates	Number of isolates (%)
Established ESBLs producers	20 (40.0)
Non ESBLs producers	30 (60.0)
Total	50

The result of the antibiotic susceptibility pattern of the confirmed ESBL-producing *E. coli* isolates is presented in Table 4.

Table 4: Antibiotic Susceptibility Profile of ESBL producing *E. coli*

Antibiotics	*E coli* (N= 20)	
	Sensitive (%)	Resistant (%)
CN (10µg)	1(5)	19(95)
CAZ (30µg)	0 (0)	20 (100)
C (30µg)	2(10)	18(90)
CTX (30µg)	0 (0)	20(100)
F (300µg)	6(30)	13(65)
CIP (5µg)	1 (5)	19(95)
AMC (30µg)	0(0)	20(100)
IPM (10µg)	20(100)	0(0)
CRO (30µg)	0 (0)	20(100)
STX (1.25/23.75µg)	1(5)	19(95)

Key: CN = Gentamicin; CAZ = Ceftazidime; CTX = Cefotaxime; F = Nitrofurantoin; C = Chloramphenical; CIP = Ciprofloxacin; AMC = Amoxicillin/clavulanic acid; IPM = Imipenem; CRO = Ceftriaxone; STX = Cotrimoxazole.

All confirmed ESBL producers (100%) exhibited resistance to ceftazidime, cefotaxime, amoxicillin-clavulanic acid (Augmentin), and ceftriaxone. In contrast, all isolates (100%) were susceptible to imipenem. High levels of resistance were also observed to gentamicin (95%; 19/20), ciprofloxacin (95%; 19/20), and cotrimoxazole (95%; 19/20), followed by chloramphenicol (90%; 18/20) and nitrofurantoin (65%; 13/20).

Twenty *E. coli* isolates that demonstrated phenotypic ESBL production and multidrug resistance were subsequently subjected to molecular characterization to evaluate the occurrence of selected ESBL-associated genes.

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The organisms were screened for *Bla*$_{SHV}$, *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$ genes. The *Bla*$_{SHV}$ gene was absent in all of the isolates whereas *Bla*$_{CTX-M}$ was present in 20(100%) of the isolates while *Bla*$_{TEM}$ was present in 16 (80%) of the isolates. The electrophoretic separation of the PCR amplicons on a 1.5% agarose gel is presented in Fig. 1.

Combined existence of *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$ was observed in most of the isolates. However, isolates represented in lanes 10, 15, 19, and 20 exhibited a single amplification band, indicating the detection of only one of the two target genes in each isolate.

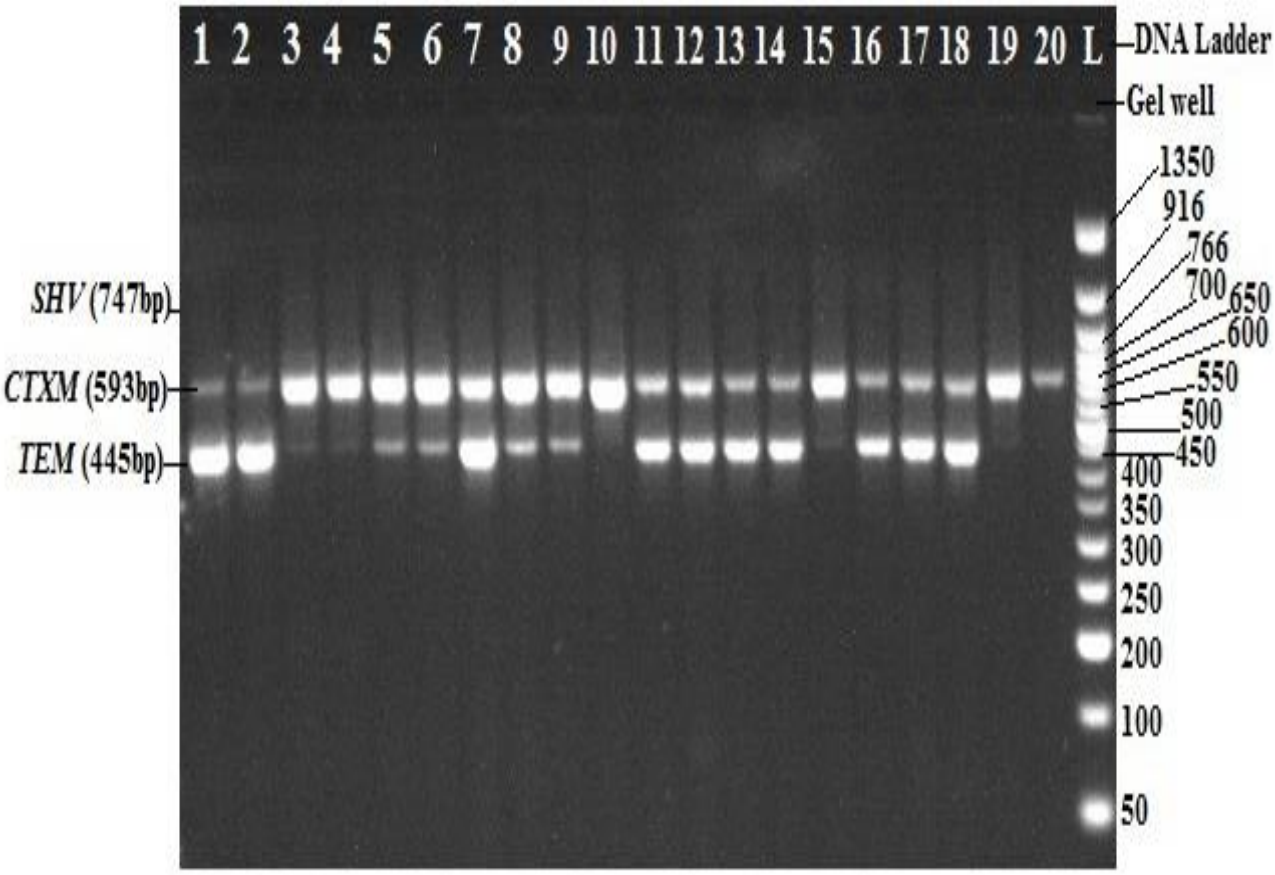


Figure 1: Multiplex PCR profile for Bla*$_{SHV}$*, Bla*$_{CTX-M}$* and Bla*$_{TEM}$* genes of *E. coli* isolates (L= 100bp marker).

Discussion

The most common uropathogen found in this investigation was *Escherichia coli*, accounting for the largest proportion of bacterial isolates. This observation agrees with findings from several earlier studies that identified *E. coli* as a major cause of urinary tract infections (Ehinmidu, 2003; El Mahmood *et al*., 2009; Akujobi and Ewuru, 2010; Pondei *et al*., 2012; Iregbu and Nwajiobi-Princewill, 2013).

Its predominance may be related to its normal association with the gastrointestinal tract and its capacity to colonize the periurethral area and subsequently gain access to the urinary tract.

The Gram-positive organisms recovered in this study included *Staphylococcus aureus, Staphylococcus saprophyticus* and *Enterococcus faecalis*, which together represented a relatively small proportion of the isolates. Although Gram-negative organisms predominated, the recovery of Gram-positive bacteria is consistent with their recognized role in UTIs. Previous studies have associated some Staphylococcus infections with urinary tract instrumentation, particularly catheterization (Moore et al., 2002; Muder et al., 2006). Differences in the distribution of Gram-positive organisms between studies may be influenced by variations in patient characteristics, clinical risk factors, healthcare exposure and antimicrobial use.

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The antimicrobial susceptibility results demonstrated substantial resistance among the *E. coli* isolates to most of the antibiotics tested. Imipenem showed the greatest activity against the isolates, whereas resistance was particularly high among several of the other antibiotics evaluated. Differences in antimicrobial susceptibility patterns have been reported among studies conducted in different geographical settings and may reflect variations in antibiotic use and local resistance patterns (Ako-Nai et al., 1993; Akujobi, 2005; Ehinmidu, 2003; El-Mahmood et al., 2009; Fadeyi et al., 2005; Ghorbani et al., 2012; Jombo et al., 2008; Oluremi et al., 2011; Raza et al., 2011; Tacconelli and Cataldo, 2008; Zalmanovici Trestioreanu et al., 2010). The high level of multidrug resistance observed in this study is consistent with the reports of resistance among uropathogenic *E. coli* from different geographical regions (Matute et al., 2004; Akram et al., 2007; Aypak et al., 2009; Muvunyi et al., 2011; Karlowsky et al., 2011).

Several factors may contribute to this pattern, including inappropriate antibiotic use and self-medication. The availability and use of substandard antimicrobial products may also contribute to treatment failure and continued selection of resistant organisms (Asadi et al., 2014; Iregbu and Nwajiobi-Princewill, 2013). Environmental exposure to antimicrobial residues may represent an additional factor in the development and dissemination of resistance. Antibiotic residues released from industrial activities can enter environmental systems, including water bodies, where they may exert selective pressure on microbial populations (Baquero et al., 2008). Inappropriate prescribing practices, particularly the use of antibiotics without prior microbiological investigation, may further promote resistance (Newman et al., 2006).

From the study carried out, 22.9% of the *E. coli* isolates were established as ESBL producers by the Double-Disk Synergy Test. This proportion was considerably lower than the percentage identified during the initial screening stage. Similar differences between screening and confirmatory ESBL tests have been described in previous studies (Giriyapur et al., 2011; Krishnamurthy et al., 2013; Yusha'u et al., 2010).

Such discrepancies may occur because phenotypic expression of ESBL activity can be affected by the existence of many β-lactamases or other mechanisms of resistance.

In comparison to values reported in a number of earlier studies, the occurrence of ESBL production was lower in this report. Studies from Pakistan, India, Latin America and other settings have documented substantially higher proportions of ESBL-producing isolates (Aminzadeh et al., 2008; Babypadmini and Appalaraju, 2004; Ejaz et al., 2011; Mekki et al., 2010; Ullah et al., 2009). Differences in prevalence between studies may arise from variations in geographical setting, study populations, specimen types, antimicrobial prescribing patterns, infection-control practices, bacterial species and laboratory methods used for ESBL detection.

The ESBL-producing isolates also demonstrated considerable resistance to both β-lactam and non-β-lactam antimicrobial agents. Resistance to gentamicin, nitrofurantoin, cotrimoxazole and ciprofloxacin indicates the occurrence of resistance extending beyond the β-lactam group. This observation is consistent with the co-resistance described among ESBL-producing organisms (Bonnet, 2004).

Molecular analysis demonstrated a high occurrence of blaCTX-M and blaTEM among the ESBL-producing isolates, while blaSHV was not detected. The predominance of blaCTX-M and the frequent detection of blaTEM alongside blaCTX-M are consistent with findings reported in previous studies of ESBL-producing organisms from patients with UTIs (Cantón et al., 2012; Harada et al., 2013; Hassan and Abdalhamid, 2014; Mohsen et al., 2016; Sharma et al., 2013; Sima et al., 2016).

The predominance of blaCTX-M may be related to the widespread dissemination of plasmid-associated ESBL determinants and the selective pressure associated with antimicrobial use. Previous studies have also linked the global spread of *E. coli* that produces the CTX-M gene to extensive use of third-generation cephalosporins, particularly cefotaxime (Seyedjavadi et al., 2016). Although blaSHV was not detected in our study, its occurrence among ESBL-producing E. coli has been documented elsewhere (Al-Marzooq et al., 2015).

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Conclusion

This study identified a 14.2% occurrence of *Escherichia coli* among urinary tract infection cases in a tertiary healthcare facility in Kano, Nigeria. A substantial proportion of the *E. coli* isolates exhibited ESBL-producing phenotypes and demonstrated elevated levels of antimicrobial resistance to cephalosporins, fluoroquinolones, and aminoglycosides, highlighting the considerable burden of antimicrobial resistance among uropathogenic *E. coli* in the study setting. Marked resistance encountered may reflect sustained antimicrobial selection pressure associated with the use of these agents. Molecular characterization revealed the predominance of blaCTX-M, with frequent co-occurrence of blaTEM and blaCTX-M genes among the ESBL-producing isolates. These findings underscore the need for strengthened antimicrobial stewardship programmes, routine surveillance of antimicrobial resistance, and rigorous enforcement of programs to prevent infection and control measures across healthcare facilities to limit the propagation and transmission of multidrug-resistant and ESBL-producing organisms.

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