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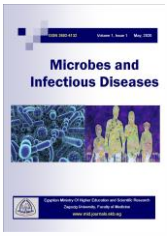

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Original article

Distribution of resistance genes encoding extended spectrum beta-lactamase (ESBL) in *Proteus mirabilis* isolated from selected hospitals in Jigawa state, Nigeria.

Samuel Olalere Obadire*[*1], Florence Oluwakemi Obadire[2], Olley Mitsan[3], Odewusi Olufunsho Odeyinka [4],. Daniel Ugbomoiko[5],. Ige Paul Ilesanmi[4], Oke Clement Olakunle [6]

1- *Federal medical center birnin kudu Jigawa state*
2- *Faculty of Agriculture, Federal University Dutse, Jigawa State*
3- *Department of Medical Laboratory Science, Igbinedion University okada, Edo State*
4- *Department of Medical Laboratory Science, Afe Babalola University Ado-Ekiti, Ekiti State (ABUAD)*
5- *department of Medical Laboratory Science, Achievers University Owo, Ondo State*
6- *Department of Microbiology, Ladoke Akintola University Ogbomoso, Oyo State*

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ABSTRACT

Background and rationale: The routine use of antibiotics in both medicine and veterinary medicine has led to widespread antibiotic resistance and development of antibiotic resistance genes, especially in Gram-negative organisms. The aim of this study was to identify the beta-lactamase *TEM, SHV* and *CTX-M* genes in clinical isolates of *Proteus* species from selected hospitals in Jigawa state, Northwest Nigeria. **Methods**: One hundred and ninety-one (191) bacterial isolates of *Proteus* species were obtained from wound swabs, urine, ear swabs, high vaginal swabs/endo-cervical swabs, sputum, aspirates from patients attending four major hospitals in Jigawa state (Hadeija General Hospital, Dutse General Hospital, Rasheed Shekoni Specialist Hospital Dutse (RSSH) and Federal Medical Centre, Birnin-kudu (FMC)) between November, 2021 to August, 2022. Resistance genes *blaTEM, blaCTX-M, blaSHV* were detected by Polymerase Chain Reaction (PCR). **Results**: Out of 191 *Proteus* species isolated 153 were *Proteus mirabilis* while 38 were *Proteus vulgaris* and 18 *Proteus mirabilis* were identify as ESBL producer using DDST. Of all the 18 *Proteus mirabilis*, 16 were positive for *Bla-TEM*, 11 *Bla-CTX-M* and 5 *Bla-SHV* genes. While 2 *Proteus mirabilis* had no resistance genes. 5(8.8%) had all the three genes. The prevalence of *Bla-TEM, Bla-CTX-M* and *Bla -SHV* genes among ESBL producing *Proteus mirabilis* isolates in this study was 84.2%, 57.9% and 26.3% respectively. However, statistics did not reveal any significant difference in expression of all the resistance genes (*Bla-TEM, Bla-CTX-M, Bla-SHV*) with respect to location of study ($p > 0.05$). **Conclusions**: This study established the presence of resistance genes in ESBL producing *Proteus mirabilis* using molecular PCR method and *Bla-TEM* and *Bla-CTX-M* were the most common. Therefore, routine laboratory detection of ESBL production in *Proteus* species is advocated.

Introduction

In recent years, there has been a sharp increase in bacterial resistance to β-lactam antibiotics, which is associated with plasmid-mediated β-lactamase propagation [1]. New extended-spectrum lactamases (ESBLs) capable of hydrolysing cephalosporins and aztreonam are part of new iterations of previous enzymes in these species [2]. Penicillins, broad-spectrum cephalosporins, and monobactams are all hydrolyzed by ESBLs, which are mainly made from TEM- and SHV-type enzymes, but may not affect

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* *Corresponding author:* Samuel Olalere Obadire
E-mail address: *obadire@gmail.com*

cephamycins and carbapenems. ESBLs are commonly found on plasmids that can be transferred between bacterial species and strains [3]. Conversely, some researchers argue that *Proteus* species rarely generate her ESBL [4]. These ESBLs are found in various Enterobacteriaceae worldwide [5]. Currently, over 200 different natural ESBL variants are known, which are responsible for resistance in an increasing variety of Gram-negative species [5]. Since β-lactams are the most commonly prescribed antimicrobial agents, the emergence of ESBL-producing bacteria in clinical infections could lead to treatment failure and pose a serious threat to current β-lactam therapy.

Proteus is a genus of Gram-negative bacteria belonging to the Enterobacteriaceae family. *Proteus* is widely distributed in the environment and is part of the normal flora of the human gastrointestinal tract. *Proteus* species is the third most common cause of nosocomial infections [6]. *P. vulgaris, P. mirabilis,* and *P. penneri* are opportunistic human pathogens [7]. *Proteus* species has long been known to be susceptible to beta-lactam antibiotics. Today, they are becoming resistant due to the spread of broad-spectrum beta-lactamases [4]. However, some researchers have reported *Proteus* species as rare ESBL producers [8, 9]. **Robert et al** [10] reported that the frequency of ESBL production was 61% in *Proteus mirabilis* and 50% in *Proteus vulgaris*. The aim of this study was to detect the ESBL producers and beta-lactamase genes *TEM, SHV,* and *CTX-M* in clinical isolates of *Proteus* species from selected hospitals in Jigawa state, Northwestern Nigeria.

Material and Methods

Study area

The bacterial strains consisted of isolates of *Proteus* species from our previous report [11]. They were obtained from patients attending four major hospitals in Jigawa state (Hadeija General Hospital, Dutse General Hospital, Rasheed Shekoni Specialist Hospital Dutse (RSSH) and Federal Medical Centre, Birnin-kudu (FMC)) between November, 2021 to August, 2022 and sample size of 191 samples was used based on the reports of 14.6% prevalence rates of *Proteus* infections in Kano [12]. The sample size was determined using the formula described by **Naing et al.** [13]. Isolates were obtained from wound swabs, urine, ear swabs, high vaginal swabs/endo-cervical swabs, sputum, aspirates from selected hospitals in Jigawa State.

Screening for suspected ESBL producers

ESBL producers were screened by disk-diffusion method using ceftazidime, cefotaxime and ceftriaxone. If the *Proteus* species isolates are resistant to any of these drugs, they are considered as suspected ESBL producers [14].

Detection of ESBL producers by Double-Disk Synergy Test (DDST)

ESBL producers were further confirmed for ESBL production by DDS test [15]. Amoxiclav disk was placed at the center of the *Proteus* species inoculated Mueller-Hinton agar plate. Third generation cephalosporins (ceftazidime and cefotaxime) were placed 15 mm apart from center of the amoxiclav disk. After incubation at 37℃ for 24 hours, a clear extension of the edge of the inhibition zone of cephalosporins disks towards amoxiclav disk was interpreted as ESBL producer. The expansion occurred because of clavulanic acid present in augmentin disc inhibited ESBL enzyme produced by the organism. Non–ESBL-producing organism (*Escherichia coli* ATCC 25922) and an ESBL-producing organism (*Klebsiella pneumoniae* ATCC 700603) were used as quality controls.

PCR methodology

Genomic DNA was extracted from the pure cultures of an overnight broth of ESBL producing *Proteus mirabilis* using the Quick-DNA Fungal/Bacterial Miniprep Kit with automated DNA extraction machine (Zybio). (Zymo Research, Catalogue No. D6005).

The quality and quantity of the extracted DNA was measured using a nanodrop (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer). The system was blanked using 1ul of DNA elution buffer. Afterwards 1ul of DNA was placed on the pedestal and measured. The concentration (ng/ul), A260/280 ratio and A260/230 ratio of the sample were subsequently recorded.

Resistance genes *blaTEM, blaCTX-M,* and *blaSHV* were detected by PCR. PCR was performed in a final volume of 50 µL using the set of primers shown in **Table (1)**. The reaction mix consist of 5× colored buffer 5 µL and 5× unshaded buffer, 3 µL of $MgCl_2$ (25 mM) (Promega, USA), 0.5 µL of dNTP (10 mM), 0.5 µL of each primer (20 mM) (Sigma Genesys) and 0.2 µL of Taq polymerase (GoTaq® G2 Flexi DNA polymerase, USA, Reference M7805) with a volume of 5 µL of DNA. PCR amplification conditions of 30 cycles of initial denaturation 94 °C for 5 min, denaturation 94 °C for

1 min, annealing at 50 °C for 1 min (*bla-TEM*) and 60 °C for 1 min (*bla-SHV* and *bla-CTX-M*), elongation 72 °C for 1 min and final elongation for 72 °C for 7 min were carried out in a thermal cycler (GeneAmp® Applied Biosystem). Amplicons were electrophoresed on 1.5% agarose gel containing TAE buffer at 135 V for 30 min (90 mMTris, 90 mM acetate, 2 mM EDTA, pH 8.0) (TAE Buffer, USA) with DNA Ladder 1 kb (Promega, USA) [16].

Ethical clearance

Ethical approval was obtained from the ethical committee of Federal Medical Centre, Birnin Kudu Hospital management and Jigawa state ministry of Health, Dutse

Data analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS® 20, USA). Descriptive statistics was used to describe the relevant variables and comparisons performed using chi-square test.

Results

Prevalence of ESBL among *Proteus* spp

Out of 191 *Proteus* species isolated, 153 were *Proteus mirabilis* while 38 were *Proteus vulgaris* and 18 *Proteus mirabilis* were identified as ESBL producer using DDST. The prevalence of *Proteus mirabilis* ESBL producing isolates was 11.8%. None of the *Proteus vulgaris* isolates in this study was found to be ESBL producers. Indeed, *Proteus mirabilis* isolates were over eleven times significantly more likely (OR=11.164, ***p***=0.018) to produce ESBL than *Proteus vulgaris* isolates as shown in **table (2)**.

Distribution of genes in extended-spectrum beta-lactamase producing *Proteus mirabilis* of all the 18 ESBL producing *Proteus mirabilis* was as follows; 16 were positive for *BlaTEM*, 11 *BlaCTX-M* and 5 *BlaSHV* genes. While 2 ESBL producing *Proteus mirabilis* show no resistance genes. 5(8.8%) had all the three genes as shown in **table (3)** and **figure (1-4)**.

Prevalence of ESBL gene among *Proteus mirabilis* isolates

The prevalence of *BlaTEM, BlaCTX-M* and *Bla SHV* genes among ESBL producing *Proteus mirabilis* isolates in this study was 84.2%, 57.9% and 26.3% respectively as shown in **table (4), figure (2, 3 and 4).**

Prevalence of ESBL genes in *Proteus mirabilis* with respect to location

With respect to the location of the study, ESBL *Proteus mirabilis* from General Hospital Hadeja and RSSH Dutse were observed to express more *Bla-TEM* gene (100%) than others from other locations. The least prevalence (50%) of *Bla-TEM* gene was recorded among ESBL producing isolates from General Hospital Dutse. However, statistics did not reveal any significant difference in *Bla-TEM* expression with respect to location of study (***p*** = 0.3050).

Bla-CTX-M gene had its highest occurrence (100%) in ESBL producing *Proteus mirabilis* from General Hospital Hadeja and RSSH Dutse. As observed with the *Bla-TEM* gene, ESBL producing *Proteus mirabilis* from General Hospital Dutse, had the least prevalence of *Bla-CTX-M* gene. However, the prevalence of *Bla CTX-M* gene, was not found to differ significantly with respect to location of study (***p***=0.082).

All ESBL producing *Proteus mirabilis* from General Hospital Hadeja did not express the *Bla-SHV* gene. However, ESBL producing *Proteus mirabilis* from RSSH were observed to have the most expression (66.7%) of the *Bla SHV* gene. Statistics failed to show any significant difference in prevalence of *Bla-SHV* gene in all study locations (***p***= 0.299) as shown in **table (5)**.

Table 1. Sequences of primers used for the analysis

ESBL genes	Primer sequence 5'-3'	Size of amplicon (bp)
blaSHV	**Forward**: ATGCGTTATATTCGCCTGTG **Reverse**: TGCTTTGTTCGGGCCAA	747
blaTEM	**Forward**: TCGCCGCATACACTATTCTCAGAATGA **Reverse**: ACGCTCACCGGCTCCAGATTTAT	445
blaCTX-M	**Forward**: ATGTGCAGYACCAGTAARGTKATGGC **Reverse**: TGGGTRAARTARGTSACCAGAAYCAGCGG	593

All primers were manufactured by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) [16].

Table 2. Prevalence of ESBL among **Proteus** spp. in the study

Bacteria	**N**	**No ESBL Pos (%)**	**OR**	**95% CI**	**P value**
Proteus mirabilis	153	18 (11.8)	11.164	0.658, 189.3	0.018*
Proteus vulgaris	38	0 (0)			

N: number of *Proteus* species screened; ESBL: extended spectrum beta-lactamase; OR: odd ratio; CI: confidence interval

Table 3. Distribution of genes in extended-spectrum beta-lactamase producing *Proteus mirabilis*

Resistance genes (ESBL)	**Total Number of Isolates Positive**	**Prevalence (%)**
BlaTEM	16	28.1
BlaCTX-M	11	19.3
BlaSHV	05	8.8
Bla-TEM+Bla-CTX-M	10	17.5
Bla-TEM+Bla+SHV	05	8.8
Bla-CTX-M+Bla-SHV	05	8.8
Bla-TEM+Bla-CTX-M+Bla-SHV	05	8.8

Table 4. Prevalence of ESBL gene among *Proteus mirabilis* isolates

Resistance genes	**N**	**No. Pos (%)**
Bla-TEM *Proteus mirabilis*	18	16 (84.2)
Bla -CTX-M *Proteus mirabilis*	18	11 (57.9)
Bla-SHV *Proteus mirabilis*	18	5 (26.3)

N: number of. ESBL positive

Table 5. Prevalence of ESBL genes in *Proteus mirabilis* with respect to location

Resistance genes	**N**	**No. Pos (%)**	***p* Value**
Bla-TEM			
FMC, BKD	8	7 (77.7)	0.3050
General Hospital, Hadeija	3	3 (100.0)	
General Hospital, Dutse	4	2 (50.0)	
RSSH, Dutse	3	3 (100.0)	
Bla -CTX-M			
FMC, BKD	8	3 (33.3)	0.082
General Hospital, Hadeija	3	3 (100.0)	
General Hospital, Dutse	4	2 (50.0)	
RSSH, Dutse	3	3(100.0)	
Bla-SHV			
FMC, BKD	8	2 (22.2)	0.299
General Hospital, Hadeija	3	0 (0.0)	
General Hospital, Dutse	4	1 (25.0)	
RSSH, Dutse	3	2 (66.7)	

FMC, BKD- federal medical centre, Birnin-kudu, RSSH-Rasheed Shekoni specialist hospital, Dutse

Fig 1. Extended-spectrum beta lactamase producers *Bla-TEM, Bla-CTX-M* and *Bla-SHV* genes detected by PCR

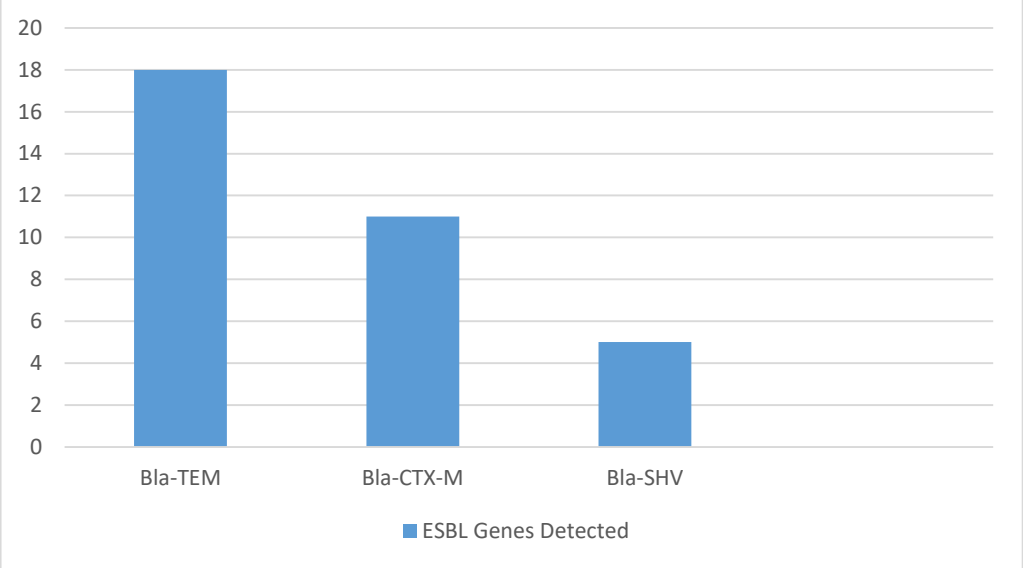
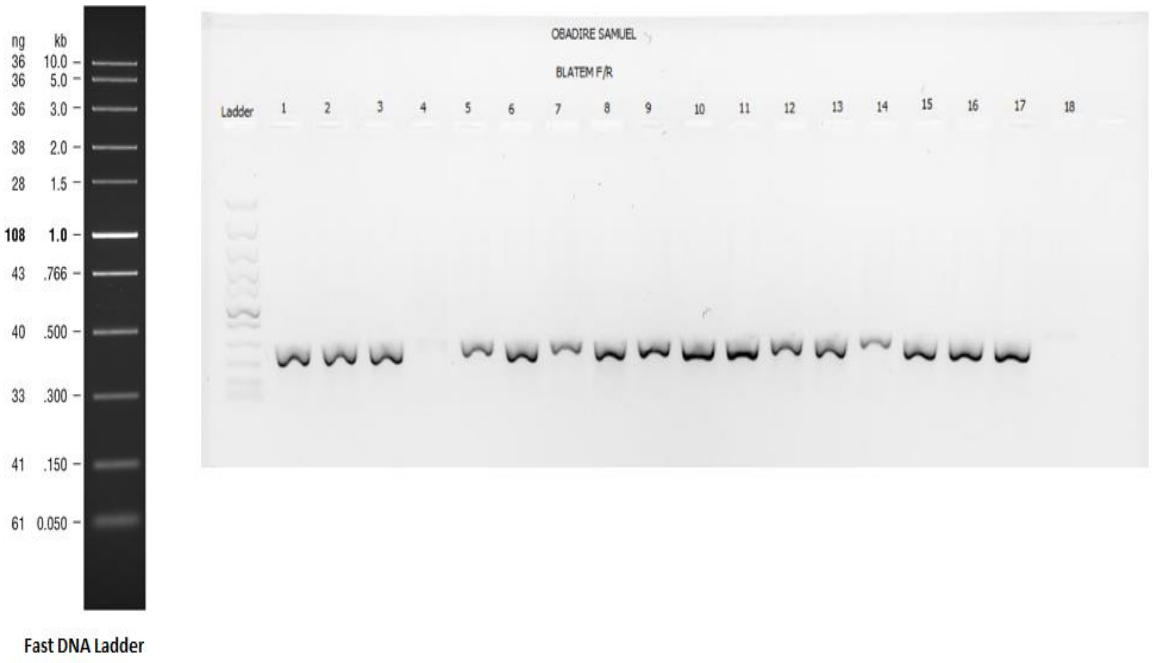
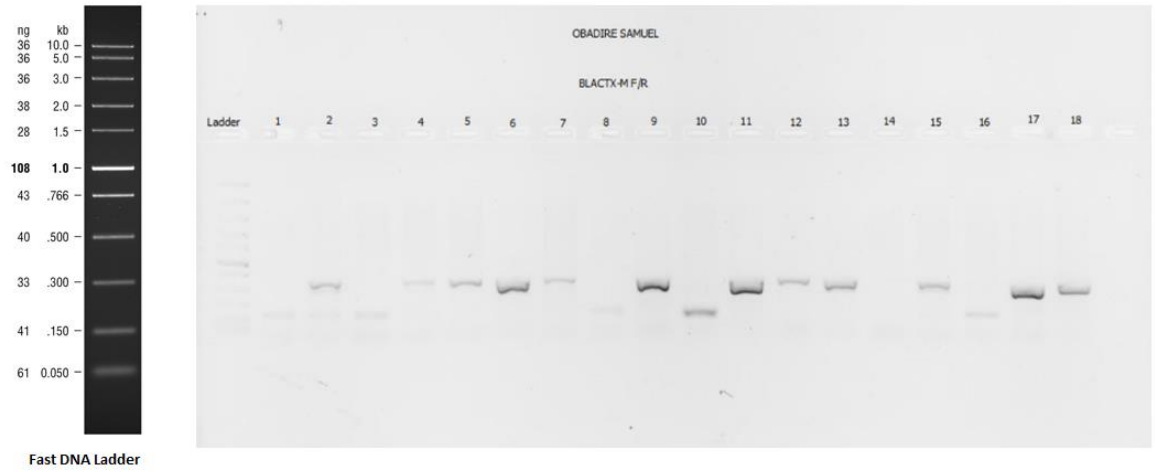


Figure 2: Agarose Gel Electrophoresis Pattern of ESBL Producing *Proteus mirabilis* (BlaTEM)



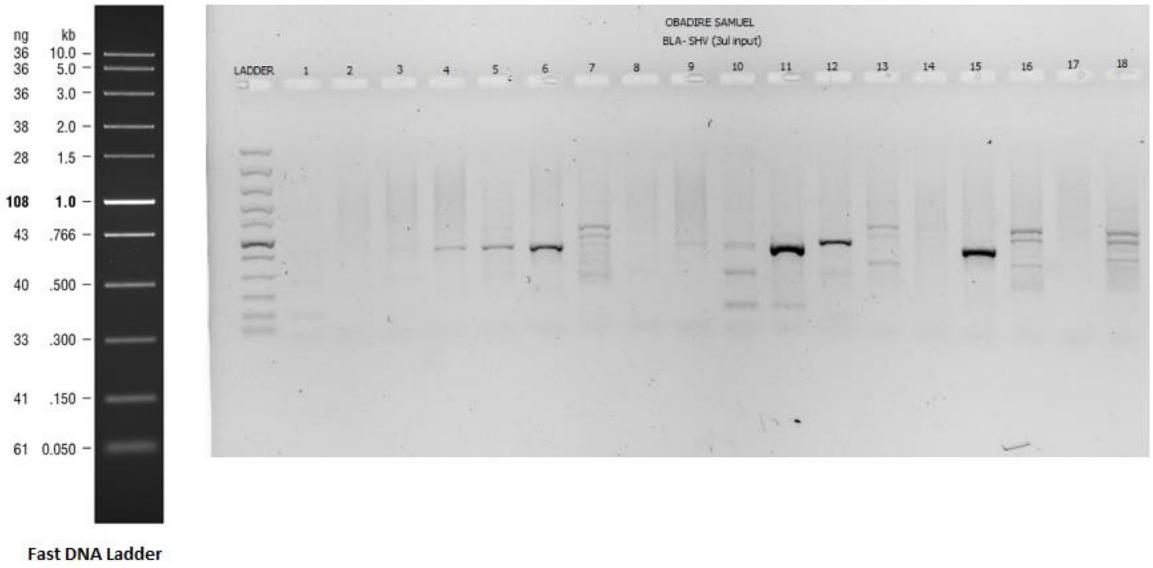
Samples 1,2,3,5,6,7,8,9,10,11,12,13,14,15,16 and 17 showed an amplicon size of 445bp corresponding to the presence of *Bla-TEM* genes.

Figure 3. Agarose gel electrophoresis pattern of ESBL producing *Proteus mirabilis* (*BlaCTX-M*)



Samples 2,5,6,7,9,11,12,13,15,17 and 18 showed an amplicon size of 593bp corresponding to the presence of *Bla-CTX-M* genes

Figure 4. Agarose gel electrophoresis pattern of ESBL producing *Proteus mirabilis (BlaSHV)*



Samples 5, 6,11,12, and 15 showed an amplicon size of 747bp corresponding to the presence of *Bla-SHV* genes.

Discussion

Antibiotic resistance of bacteria is commonly seen in daily medical practice with multi-drug resistant Gram negative bacteria posing the greatest challenges to public health [17]. In this study, 16 (50%), 11(34.4%) and 5(15.6%) *Proteus mirabilis* isolates harboured *Bla-TEM*, *Bla-CTX-M* and *Bla-SHV* genes respectively and this is in agreement with most reports of other researchers [18, 19 20]. The genotype *bla-TEM/bla-CTX-M* in this study had the highest frequency among the multiple ESBL genes produced by the *Proteus mirabilis* and this was in line with the work of [21, 22, and 23]. The detection of more than single ESBL gene in some of *Proteus mirabilis* in the present study is in consonance with the research work of [24], where they observed the presence of multiple ESBL genes (*bla-SHV and bla-TEM*) in enterobacteriaceae. The high occurrence frequencies of *bla-TEM* and *bla-CTX-M* genes in this study agreed with the work of [25] who observed that *blaTEM* and *blaCTX-M* genes were the commonest genotype of ESBL genes among human population, while the strains harbouring *bla-SHV* genes were also known to cause infections in some cases. Furthermore, the results of our study is in agreement with the outcome of [19] who reported the prevalence of *bla-TEM* and *bla-SHV* to be 65.5% and 15% respectively; and 14 isolates (19.0%) were observed to harbour both *blaTEM* and *blaSHV* genes

among ESBL-producing *Proteus mirabilis*. **Omar et al.**, [26] reported that the most frequent prevalent ESBL genes among enterocbacteriaceae was *blaCTX-M* gene, followed by *blaTEM*, while the least was *blaSHV* gene. The detection of *TEM*, *CTX-M* and *SHV* genes in this study were confirmed in 88.9% of the ESBL-producing *Proteus mirabilis* and this agreed with the findings of research work by [27]. Some studies have shown prevalence of ESBL-producing enterobacteriaceae in hospitals in African countries including Algeria, Morocco, South Africa and Nigeria [28, 29 30, 31, 32].These reports are similar to the findings of this present study.

Obviously, this is the first molecular study on ESBL-producing *Proteus* species in Jigawa state, Nigeria. Co-existence of different beta-lactamase genes in clinical isolates has been reported by several investigators elsewhere. Our findings showed that over half of the ESBL-producing *Proteus mirabilis* were molecularly confirmed to have multiple ESBL genes. The most prevalent ESBL genotype among the isolates was *bla-TEM* and *bla-CTX-M*. This could mean that *Proteus mirabilis* are rapidly becoming ESBL-producer and habouring multiple ESBL genes, *Bla-TEM* and *bla-CTX-M*, in Jigawa state, Nigeria. Therefore, routine detection and confirmation of ESBL-producing bacterial strains is very pertinent to assist in the choice of the best effective antibiotics for the treatment and management of bacterial infections. Effective infection control measures, continuous surveillance, rational use of drugs and routine clinical detection of ESBL-producing bacteria are necessary to curtail serious public health problems in this environment.

Conclusion

These results demonstrate that more than half of ESBL-producing *Proteus mirabilis* are molecularly confirmed to carry multiple ESBL genes. The highest ESBL genotypes among isolates were *blaTEM* and *blaCTX-M*. This may imply that *Proteus mirabilis* is rapidly becoming ESBL producer and that the state of Jigawa in northwestern Nigeria contains multiple ESBL genes. Judicious use of antibiotics is advocated to curb this trend.

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