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Original Article

Prevalence, Antibiotic Susceptibility Pattern and Detection of Transferable Resistant Genes in *Proteus Species* from Urinary Tract Infections in a Tertiary Hospital in South-East of Nigeria

Orjioke Nkemdilim Nwaka[1*] and Onyemelukwe Ngozi Franca[1]

[1]Department of Medical Laboratory Sciences, University of Nigeria, Enugu Campus, Nsukka, Nigeria

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***Corresponding Author:**

Orjioke Nkemdilim Nwaka

Department of Medical Laboratory Sciences, University of Nigeria, Enugu Campus, Nsukka, Nigeria

nkemorjioke1@gmail.com

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ABSTRACT

Drug-resistant *Proteus species* cause global public health threats, including in Nigeria, due to antibiotic resistance. **Objective:** To determine the prevalence, antibiotic susceptibility, and detection of resistant genes in *Proteus species* causing UTIs in a Nigerian hospital. **Methods:** A cross-sectional study was conducted over seven months at Alex-Ekwueme Federal University Teaching Hospital in Abakaliki, Ebonyi State, Nigeria. The study included 650 urine samples from male and female in-patients and out-patients displaying UTI symptoms. Disc diffusion method was used for antimicrobial susceptibility testing and double disc-synergy test was employed to check for the presence of extended spectrum beta-lactamases. Polymerase chain reaction (PCR) was utilized to screen for transferable resistant genes and mobile genetic elements. **Results:** Out of 650 urine samples, 84 (12.9%) *Proteus species* isolates were identified. 60 (71.4%) were Proteus mirabilis and 24 (28.6%) were Proteus vulgaris. Females had a higher distribution of isolates (76.2%) compared to males (23.8%) (p=0.010). Age group showed higher isolates in the 31-40 (23.8%) and 41-50 (22.6%) age groups (p<0.001). No significant association was found between *Proteus species* and urine types or patient categories (p=0.061 and p=1.000, respectively). Levofloxacin and ceftazidime exhibited the greatest effectiveness, while nalidixic acid, imipenem, and nitrofurantoin displayed the highest resistance against *Proteus species*. 56% of Proteus isolates were multidrug resistant. PCR analysis detected TEM (23.1%), CTX-M (23.1%), SHV (15.4%), aab(61)-1b (10.3%), qnrB (2.6%), and class 1 integrase gene (25.7%). **Conclusions:** Proteus isolates carry transferable resistant genes associated with class 1 integrase.

INTRODUCTION

Proteus species, a motile, facultative anaerobic Gram-negative rod, belongs to the Enterobacteriaceae family. The genus includes P. mirabilis, P. vulgaris, P. penneri, P. myxofaciens, and P. hauseri, along with three unnamed genomospecies and 80 O-antigenic serogroups [1]. Swarming behavior is a notable characteristic of *Proteus species* [2]. They are commonly found in the intestinal tract of humans and animals, as well as in water, sewage, and soil [3]. *Proteus species* cause various infections, including urinary tract infections (UTIs), wound infections, and occasionally respiratory tract infections, otitis media, eye infections, bacteremia, and sepsis [1, 4, 5]. *Proteus species* possess virulence factors such as fimbriae and adhesion molecules for uroepithelium adherence [6]. They also have flagella for motility and swarming growth, which contributes to kidney stone formation. Urease production aids in colonization, stone formation, urinary catheter obstruction, and recurrent infections. Additionally, *Proteus species* generate cytotoxic hemolysins, biofilms for antibiotic protection, toxic agglutinins for cell aggregation and cytotoxicity, and proteases for antibody degradation [7, 8]. Antibiotics including benzylepenicillin, oxacillin, tetracycline, macrolides, and nitrofurans are naturally resistance in Proteus species [4]. Plasmid-mediated beta-

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lactamases in *Proteus species* can make them resistant to beta-lactam antibiotics. In recent years, there have been documented cases of *Proteus species* producing extended spectrum beta-lactamases (ESBLs), which makes treating urinary tract infections (UTIs) increasingly difficult due to widespread antibiotic resistance. This resistance extends to other antibiotic families like aminoglycosides and quinolones. In Nigeria, the most commonly used antibiotics for treating UTIs and other infections are third-generation cephalosporins, fluoroquinolones, and aminoglycosides. However, resistance to these antibiotics among *Enterobacteriaceae*, including *Proteus species*, is on the rise. Much work has been done on *Proteus species* but not on the molecular aspect of it in the South-East of Nigeria.

This study aimed to assess the frequency and pattern of antibiotic susceptibility, as well as detect antibiotic-resistant genes in urine samples from *Proteus species* at a tertiary hospital in southeastern Nigeria. Such research is crucial for informing antibiotic policies and controlling resistance in *Proteus species*.

METHODS

A seven-month cross-sectional study was conducted at Alex-Ekwueme Federal University Teaching Hospital in Abakaliki, Ebonyi State, Nigeria, from September 1, 2022, to April 1, 2023. The study included a random sample of in-patients and out-patients of all age groups, both males and females, who attended the hospital during this period. The study was ethically approved by the Research and Ethics Committee of Alex-Ekwueme Federal University Teaching Hospital Abakaliki, Ebonyi state, Nigeria (Approval number: AE-FUTHA/REC/VOL3/2022/070). Approval was granted from 15th June 2022 to 14th June 2023. Six hundred and fifty (650) urine samples were used for this study. Fisher's method (N = Za2P (1-P)/D2) was used to determine the minimal sample size. A score of 1.96 for the 95 percent confidence interval, P for prevalence, and D for allowable error were provided (5 percent). 21.3% prevalence was used for this study. The fisher's formula, N = Za2P (1-P)/D2, was used to calculate the sample sizes.

Where;

Za =significant level set at 95% confidence level. Za is 1.96 for a two-tailed test.

P = prevalence of the attribute under study. P is 21.3 % (0.213)

D = margin of error tolerated. D is 5% (0.05)

N = minimum sample size =Za2P(1-P)/D2

Substituting in the formula,

N = 1.962 X0.213(1-0.213)/(0.05)2= 258 approximately.

Allowing 10% non –responses, N= 10 X 258/100 = 25.8

N = 258+ 25.8 = 284

Patients provided consent before sample collection. A questionnaire gathered data on patient demographics, UTI symptoms, antibiotic use, and patient category. Sterile universal containers with boric acid were used for sample collection. Participants were instructed on how to collect early morning midstream urine. For in-patients with urinary catheters, urine was collected with a syringe and transferred to a sterile container. Willing participants with UTI symptoms and no recent antibacterial therapy within two weeks prior to the hospital visit were included in the study. The bacteria were isolated and identified at the hospital's Microbiology unit. Urine samples were cultured on blood agar and cysteine lactose electrolyte deficient agar, and then incubated at 37°C for 24 hours. The isolates were identified based on morphology, swarming on blood agar, Gram stain reaction, and biochemical tests. The Proteus isolates underwent further stages, including antimicrobial susceptibility testing, detection of extended spectrum beta-lactamases using polymerase chain reaction, and gel electrophoresis. Antimicrobial susceptibility was assessed using the disc diffusion method according to Clinical and Laboratory Standards Institute protocols [9]. Proteus isolates cultured overnight were adjusted to 0.5 McFarland turbidity and Mueller Hinton agar plates were inoculated with the test organisms using sterile swabs. Antibiotic discs were placed on the plates using sterile forceps. The plates were then incubated at 37°C for 18 hours, and the resulting zone of inhibition was measured and recorded. The zones of inhibition were categorized as sensitive, intermediate, or resistant based on the Clinical and Laboratory Standards Institute guidelines [9]. The antimicrobial discs used included ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefixime (5 µg), cefuroxime (30 µg), augmentin (30 µg), levofloxacin (5 µg), ofloxacin (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), nitrofurantoin (300 µg), gentamicin (10 µg), and nalidixic acid (30 µg). Extended spectrum beta-lactamases detection employed ceftazidime, cefotaxime, and ceftriaxone. Positive ESBL production was indicated by ≤ 22 mm for cefotaxime, ≤ 17 mm for ceftazidime, and ≤ 19 mm for ceftriaxone [9]. Using the double disc-synergy test [10], a 30 µg augmentin disc (Oxoid, UK) was placed at the center of a Mueller Hinton agar plate. Surrounding the augmentin disc, three discs containing 30 µg each of ceftazidime, ceftriaxone, and cefotaxime were positioned at a distance of 30 mm from the center disc. The plate was incubated at 37°C for 24 hours. If the inhibitory zone extended towards the augmentin disc, it indicated favorable evidence for ESBL synthesis. Multidrug resistant isolates, which are resistant to more than three antibiotic classes, were screened for the following genes blaTEM, blaSHV, blaCTX-M, qnrA, qnrB, aac(6')-Ib genes and class1

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and 2 intigrase genes by PCR. Bacterial DNA was extracted using the Thermo Scientific GeneJET Genomic DNA Purification Kit. The PCR reaction mixture consisted of 12.5µl of 2 X Master mixes with standard buffer, 0.5µl each of forward and reverse primers (0.2µM), 3µl of extracted DNA (0.057µg), and 8.5µl of sterile nuclease-free water, making a total volume of 25µl. The Taq Quick Load 2X Master Mix with Standard Buffer (New England Biolabs, MA, U.S.A.) used for the PCR. The mixture was vortexed, placed in a thermal cycler machine, and PCR was performed according to the primer and cycling conditions given in Table 6 supplementary material. The resulting PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide (1µg/mL). Electrophoresis was carried out at 110 volts for 45 minutes, and the gel was visualized under an ultraviolet transilluminator. A 100 bp DNA ladder (New England Biolabs, USA) was used as a molecular weight marker. Version 22.0 of SPSS for Windows was used for all statistical analyses. For the purpose of describing categorical variables, descriptive statistics (% and frequencies) were used. With a 95% confidence interval, the Pearson Chi-square ($\chi 2$) test was used to identify significant changes in proportions. A p-value less than 0.05 specified a connection that was statistically significant.

RESULTS

A total of 84 (12.9%) isolates of *Proteus species* of which 60 (71.4%) and 24 (28.6%) isolates were Proteus mirabilis and Proteus vulgaris respectively were isolated from the 650 urine samples analyzed. The association between prevalence of *Proteus species* and types of urine were not statistically significant (p=0.061, X2 (df) = 5.586 (2)). Also, the association between prevalence of *Proteus species* and patients' category were not statistically significant (p=1.000, X2 (df) < 0.001 (2)). The association between prevalence of Proteus species and gender were statistically significant (p=0.010, X2 (df) = 9.157(2)). Also, the association between prevalence of *Proteus species* and age group were statistically significant (p < 0.001, X2 (df) = 63.015(14))(table 1).

Table 1: Distribution of Proteus isolates in relation to Demographic characteristics of patients

Demographic Characteristics	*Proteus species*		Total	x^2	p-value
	Proteus mirabilis	*Proteus vulgaris*			
Urine type					
Catheter urine (n=22)	5 (100%)	0 (0%)	5 (6.0%)	5.586	0.061
Non-catheter urine (n=628)	55 (69.6%)	24 (30.4%)	79 (94.0%)		
Total	60 (71.4%)	24 (28.6%)	84 (100%)		
Patient category					
In-patient (n=216)	20 (71.4%)	8 (28.6%)	28 (33.3%)		
Out-patient (n=434)	40 (71.4%)	16 (28.6%)	56 (66.7%)	<0.001	1.000
Total	60 (71.4%)	24 (28.6%)	84 (100%)		
Gender					
Male (n=250)	13 (65.0%)	7 (35.0%)	20 (23.8%)	9.157	0.010
Female (n=400)	47 (73.4%)	17 (26.6%)	64 (76.2%)		
Total	60 (71.4%)	24 (28.6%)	84 (100%)		
Age groups (years)					
1 - 10 (n=91)	3 (50%)	3 (50%)	6 (7.1%)	63.015	<0.001
11 - 20 (n=98)	5 (100%)	0 (0%)	5 (6%)		
21 - 30 (n=151)	4 (50%)	4 (50%)	8 (9.5%)		
31 - 40 (n=146)	15 (75%)	5 (25%)	20 (23.8%)		
41 - 50 (n=77)	14 (73.7%)	5 (26.3%)	19 (22.6%)		
51 - 60 (n=40)	7 (77.8%)	2 (22.2%)	9 (10.7%)		
61 - 70 (n=33)	11 (78.6%)	3 (21.4%)	14 (16.6%)		
71 - 80 (n=14)	1 (33.3%)	2 (66.7%)	3 (3.6%)		
Total	60 (71%)	24 (29%)	84 (100%)		

n = number tested

Table 2 demonstrates that Levofloxacin and ceftazidime showed the highest activity against the *Proteus isolates* while nalidixic acid and nitrofurantoin exhibited the highest resistance against Proteus isolates. Fifty six percent (47/84) of the Proteus isolates were multidrug resistant. (Table 4 supplementary material) There was no significant difference in the number of ESBL producers between the MDR isolates of Proteus species (p = 0.920, X2 = 0.010 (1)) (Table 5 supplementary material).

Table 2: Antibiotic Susceptibility Profile of *Proteus Species* Isolates

ANTIBIOTICS	Disc conc.	*Proteus mirabilis*			*Proteus vulgaris*		
		Sensitive (%)	Intermediate (%)	Resistant (%)	Sensitive (%)	Intermediate (%)	Resistant (%)
Levofloxacin	LEV (5ug)	48 (80%)	7 (11.7%)	5 (8.3%)	14 (58.3%)	2 (8.3%)	8 (33.3%)
Cefazidime	CAZ (30ug)	38 (63.3%)	7 (11.7%)	15 (25%)	15 (62.5%)	3 (12.5%)	6 (25%)
Ceftriaxone	CTR (30ug)	31 (51.7%)	12 (20%)	17 (28.3%)	12 (50%)	4 (16.7%)	8 (33.3%)
Ofloxacin	OFL (5ug)	28 (46.7%)	9 (15%)	23 (38.3%)	13 (54.2%)	5 (20.8%)	6 (25%)
Ciprofloxacin	CPR (5ug)	28 (46.7%)	6 (10%)	26 (43.3%)	11 (45.8%)	3 (12.5%)	10 (41.7%)
Gentamicin	GEN (10ug)	18 (30%)	12 (20%)	30 (50%)	10 (41.7%)	4 (16.7%)	10 (41.7%)
Cefixime	CXM (5ug)	18 (30%)	13 (21.7%)	29 (48.3%)	10 (41.7%)	1 (4.2%)	13 (54.2%)
Cefuroxime	CRX (30ug)	15 (25%)	6 (10%)	39 (65%)	9 (37.5%)	4 (16.7%)	11 (45.8%)
Cefotaxime	CTX (30ug)	16 (26.7%)	5 (8.3%)	39 (65%)	2 (8.3%)	2 (8.3%)	20 (83.3%)

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Augumentin	AUG (20ug)	10 (16.7%)	6 (10%)	44 (73.3%)	5 (20.8%)	1 (4.2%)	18 (75%)
Imipenem	IMP (10ug)	13 (21.7%)	5 (8.3%)	42 (70%)	1 (4.2%)	1 (4.2%)	22 (91.7%)
Nacidixic acid	NA (10ug)	12 (20%)	2 (3.3%)	46 (76.7%)	2 (8.3%)	0 (0%)	22 (91.7%)
Nitrofuran-toin	NIT (300g)	3 (5%)	8 (13.3%)	49 (81.7%)	2 (8.3%)	4 (16.7%)	18 (75%)

For different gene types of P. mirabilis and P. vulgaris, frequency is given in table 3.

Table 3: Gene Frequency of the *Proteus Species*

Gene type	Specific Gene	*Proteus species*		Total frequency (%)
		P. mirabilis	*P. vulgaris*	
ESBL gene	**TEM**	7	2	9 (23.1)
	SHIV	4	2	6 (15.4)
	CTX-M	7	2	9 (23.1)
PMQR gene	**QnrA**	0	0	0 (0)
	QnrB	1	0	1 (2.6)
AMR gene	**aab(b) – 1b**	2	2	4 (10.3)
MGE	**INT1**	7	2	10 (25.7)
	INT2	0	0	0 (0)
Total		29	10	39 (100)

MDR: Multidrug resistant strain, ESBL: Extended Spectrum Beta-Lactamase genes.PMQR: Plasmid Mediated Quinolone Resistant gene, AMR: Aminoglucosides Resistant gene (aab (6')-1b), MGE: Mobile Genetic Element (INT1: class1integrase and INT2: class2 integrase).

PCR revealed the presence of genes: TEM, CTX-M, SHV, aab (61)-1b 4 (10.3%), qnrB 1 and class1 integrase gene. QnrA gene and class 2 integrase gene were not detected. The entire screened DNA, except the DNA loaded in lane 4, were positive for the Bla TEM ESBL Gene.

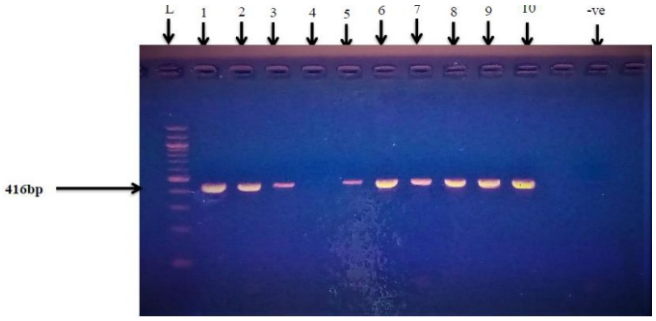

Figure 1: BlaTEM gene (41bp) gel image

(key: L = DNA ladder; Number 1 – 10 = Different Proteus isolates analyzed with PCR; -ve= Negative control)

The entire screened DNA, except the DNA loaded in lane 7, were positive for the Bla CTX-M gene. The DNA loaded in lanes 1, 4, 5, 7, 8, and 9 were positive for Bla SHV gene, while the DNA loaded in lanes 2,3,6 and 10 were negative for the Bla SHV gene.

The other unaccounted bands in the gel could as a result of primer dimmers.

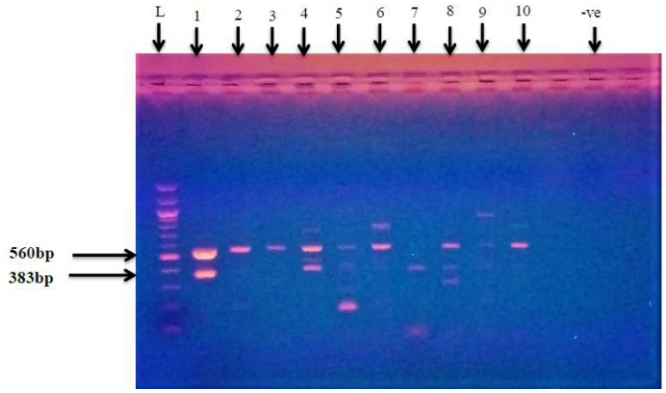

Figure 2: Figure 2: CTX-M gene (560bp) and SHV gene (383bp) gel image.

(key: L = DNA ladder; Number 1 – 10 = Different Proteus isolates analyzed with PCR; -ve= Negative control)

Only the DNA loaded in lane 1 was positive for the QnrB Gene. The DNA loaded in lanes 2, 8, 9 and 10 were positive for AAC (6)-Ib (figure 3).

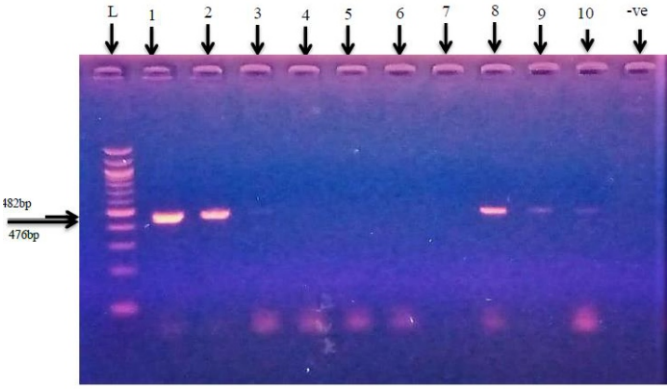

Figure 3: QnrB Gene (476bp) and AAC (6')-Ib (482bp) gel image

All the screened DNA were positive for the IntI-1gene as shown in figure 4.

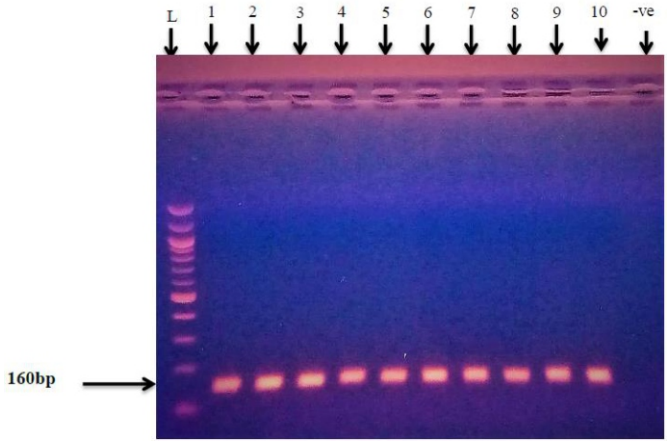

Figure 4: Figure 4: IntI-1 gene (160bp) gel image

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DISCUSSION

Among the 84 Proteus isolates, two species were identified: *Proteus mirabilis* and *Proteus vulgaris*. *P. mirabilis* was the most commonly isolated species, accounting for 71.4% of the isolates, while P. vulgaris accounted for 28.6%. It is consistent with previous research that *P. mirabilis* is the most commonly isolated *Proteus species*. *P. mirabilis* is known to be a major cause of kidney stone-related infections, a serious complication of unresolved or recurrent bacteriuria. Out-patients had a higher number of Proteus isolates compared to in-patients. Among the Proteus isolates from catheterized urine, all were *P. mirabilis*. Female patients had a higher number of Proteus isolates (76.2%) than male patients (23.8%), which aligns with previous research. In Olowe et al., report in South-West part of Nigeria female patients recorded 76.9% while male patients recorded 23.1% [11]. In a study performed by Khanal *et al.*, in Nepal female patients recorded 64.9% while male patients recorded 15.9% [12]. Similarly, according to Ahmed et al., female patients recorded 73% while male patients recorded 27% [13]. The shorter female urethra and its proximity to the rectum may make it easier for bacteria to enter the urinary system, increasing the risk of urinary tract infections (UTIs) in female patients [14]. The most affected age groups were patients aged 31-40 years and 41-50 years, which correspond to sexually active age groups. This is consistent with research conducted in Nigeria, Ethiopia, and Northern India [11, 15, 16]. Treating Proteus urinary tract infections has become increasingly challenging due to the emergence and widespread spread of antibiotic resistance. In this investigation, the most effective antibiotics against *P. mirabilis* and *P. vulgaris* were ceftazidime and levofloxacin. However, P. mirabilis isolates showed less resistance to antibiotics compared to P. vulgaris. The Proteus isolates exhibited decreased susceptibility to antibiotics such as ceftriaxone, ofloxacin, gentamicin, and ciprofloxacin, and high resistance to cefotaxime, cefixime, nalidixic acid, Augmentin, cefuroxime, imipenem, and nitrofurantoin. *Proteus species* are naturally resistant to nitrofurantoin and imipenem [4]. Multidrug resistance was observed in the majority of Proteus isolates, with *P. vulgaris* showing a higher level of multidrug resistance than P. mirabilis. This aligns with the finding that *P. vulgaris* isolates exhibited higher levels of antibiotic resistance compared to P. mirabilis isolates. Multidrug resistance was observed across both male and female isolates, in both in-patient and out-patient isolates, and across all age groups. The high prevalence of multidrug resistance in Proteus isolates from this study confirms earlier reports of increasing multidrug resistance in Nigeria among Proteus species and other members of the Enterobacteriaceae family [17-19]. Antimicrobial resistance is a significant threat to global public health. The World Health Organization reports that 4.95 million deaths worldwide in 2019 were attributed to bacterial resistance to antibiotics, with Western Sub-Saharan Africa having the highest mortality rate [20]. The prevalence of ESBL production in *Proteus species* and other Enterobacteriaceae has been reported to be increasing in Nigeria [17-19]. Molecular analysis revealed the presence of various resistance genes in the Proteus isolates, including ESBL genes (TEM, SHV, and CTX-M), aminoglycoside-resistant gene (aab (6')-1b), plasmid-mediated quinolone resistance gene (qnrB), and mobile genetic element (class 1 integrase). TEM and CTX-M genes were the most frequently detected each with a frequency of 9 (23.1%). The qnrA gene was not detected in this study. Class 1 integrase was present in all tested samples, while class 2 integrase was absent. The detected plasmid-mediated resistance genes were associated with class 1 integrase. ESBL genes, especially those from the CTX-M group, are the most prevalent and widely spread among Proteus isolates. In the United States and Europe, CTX-M genes have been found to be the most commonly occurring ESBL genes in P. mirabilis [21-23]. Quinolone resistance genes (qnrB) were not highly prevalent in this study, consistent with findings from other studies in Turkey, Argentina, and Egypt [24, 25]. Girlich states that the qnrA gene in Proteus is still very uncommon, with just one isolate out of 1,468 known to produce the gene [26, 27]. The aab (6')-1b gene, associated with aminoglycoside resistance, had a frequency of 10.3% in this study. Ogbulu et al., recorded a prevalence of 17.2%, while Alabi et al., recorded a higher prevalence of 33.3% [17, 18]. The presence of multiple resistant genes associated with class 1 integrase suggests that the accumulation of resistance determinants through mobile genetic elements contributes to the observed multidrug resistance in Proteus isolates.

CONCLUSIONS

Proteus isolates showed multidrug resistance and reduced sensitivity to tested antibiotics. Plasmid-mediated resistant genes (TEM, SHV, CTX-M, aab (61)-1b, qnrA) were detected, indicating increased antibiotic resistance. Each isolate carried multiple resistant genes associated with class 1 integrase. Early disease diagnosis, reduced antibiotic exposure, and immunization can help curb antibiotic resistance spread.

Authors Contribution

Conceptualization: ONN
Methodology: ONN
Formal analysis: ONN

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Writing-review and editing: ONN, ONF
All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Source of Funding

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