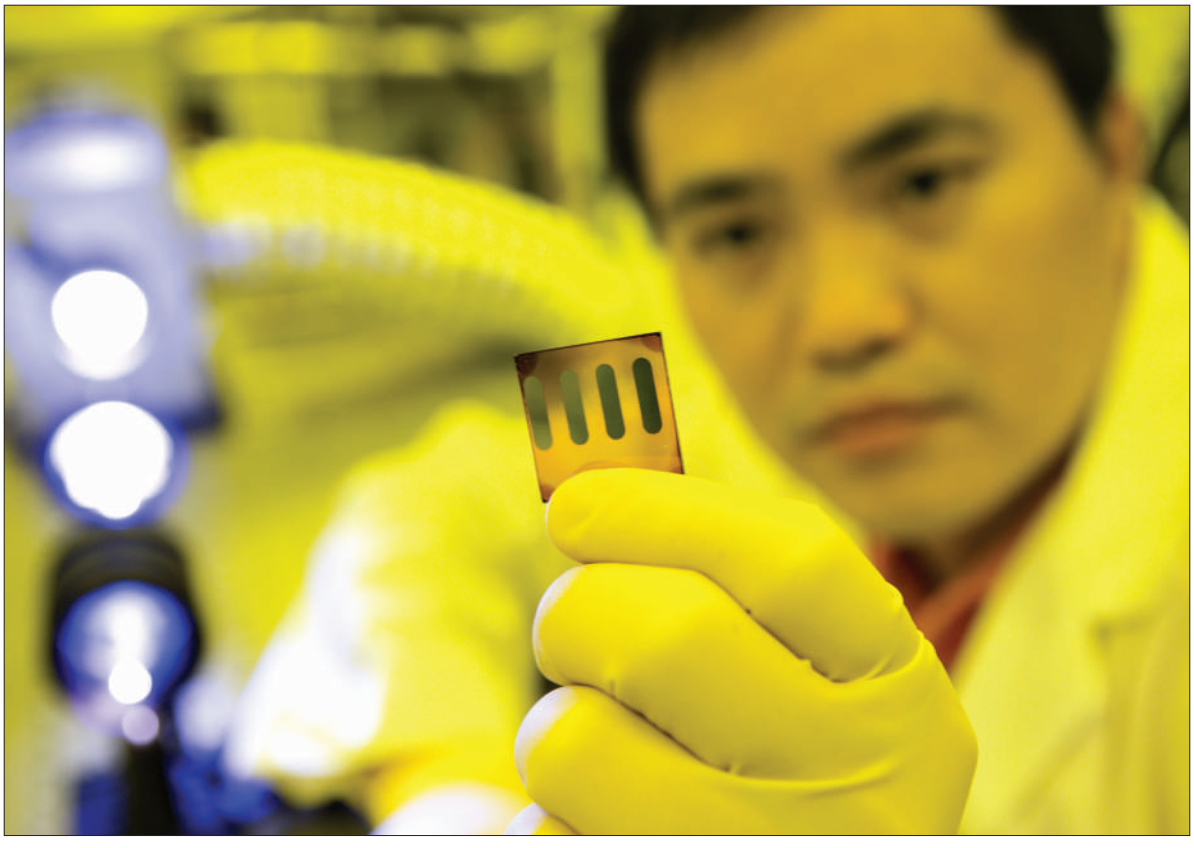

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Research Article
Molecular Characterization of *Pseudomonas aeruginosa* Isolated from Patients with Chronic Respiratory Diseases in Ile-ife, Nigeria

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Abstract

Background and Objective: Respiratory infections are common occurrence in patients with chronic respiratory diseases. The infections are of different severity and caused by different organisms. *Pseudomonas aeruginosa* is a major risk factor for death in chronic respiratory diseases. The current study was designed to assess the phenotypes, molecular characteristics and prevalence of *Pseudomonas aeruginosa* isolated from patients with chronic respiratory diseases in Ile-Ife, South Western Nigeria. **Materials and Methods:** Adult patients with respiratory diseases were recruited between September, 2014 and June, 2015 and their sputum samples collected for culture by streak plate method at 37°C. Antimicrobial susceptibility test was subsequently carried out and the molecular characterization was done by the polymerase chain reaction using appropriate primers. **Results:** Thirty-one adult patients were recruited with a mean (SD) age of 62.2 years (+25.8). Twelve (38.8%) *Pseudomonas aeruginosa* isolates were recovered from sputum samples of twelve patients. Resistance was very pronounced to most of the antibiotics tested particularly including amoxicillin 100%, augmentin 100%, cefuroxime 100% and piperacillin 100%. However, the isolates were mostly sensitive to ceftazidime and gentamicin (8.3%). The beta lactamase resistance gene, bla_{CTX-M} was detected in 8 (66.7%) of the isolates while 5 (41.7%) had bla_{SHV-1} gene and 5 (41.7%) harboured bla_{TEM-1} gene. **Conclusion:** The presence of bla_{CTX-M}, bla_{SHV-1} and bla_{TEM-1} in the isolates may be partly responsible for the high rate of resistance to the beta lactams antibiotics and ceftazidime, gentamicin, imipenem and ofloxacin, were the most active antibiotics against the *Pseudomonas aeruginosa* isolates.

Key words: *Pseudomonas aeruginosa*, respiratory diseases, antibiotics, resistance genes, beta lactamase, infections, antimicrobial susceptibility

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chronic respiratory diseases are respiratory diseases affecting the structures of air passage ways (trachea and bronchi) and parts of the lung (alveoli and the interstitial) which include; asthma, chronic obstructive pulmonary disease, chronic bronchitis, cystic fibrosis, emphysema and are sometimes associated with chronic cough and sputum production for at least three months for two consecutive years[1]. They are also characterized by repeated and long standing symptoms; including infections and hypoxemia. Infection is a key complication causing further destruction in lung capacity and reduction in oxygen level of the blood. Globally, chronic respiratory infections are among the leading causes of morbidity and mortality[2].

Organisms commonly implicated in infections of chronic respiratory diseases include; *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Morazella catarrhalis* and *Pseudomonas aeruginosa*[3-6]. Among the bacteria commonly implicated in chronic respiratory diseases, *Pseudomonas aeruginosa* is a major risk factor for death; perhaps for its role in the decline of lung functions[7], its association with severe and acute pulmonary exacerbations[8] and as a prognostic marker for mortality among hospitalized patients[4], with chronic obstructive pulmonary diseases. Significantly, a mortality of 58.6% was reported in a study of chronic obstructive pulmonary disease patients[4] with *Pseudomonas aeruginosa* isolated from their sputum as against 34.9% in similar patients but with no case of *Pseudomonas aeruginosa* in their sputum. Also chronic *Pseudomonas aeruginosa* infections have been found to be associated with inflammation levels in the airways and in the lung[9]. Therefore, the current study was designed to determine the phenotypes and molecular characterization of *Pseudomonas aeruginosa* isolated from patients with chronic respiratory diseases in Ile-Ife, Nigeria.

MATERIALS AND METHODS

Sample collections: The study was conducted between September, 2014 and June, 2015 at the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria and the Department of Medical Microbiology, Ladoke Akintola University of Technology, Oshogbo, Nigeria. Sputum samples from thirty-one adults (in and out patients) with respiratory diseases were collected inside sterile wide mouth sampling bottles. The patients in most cases with persistence cough (>3 months) were confirmed by the physician. Respiratory-based questionnaire was also used to collect their demographic information and medical history. The patients were recruited from the Obafemi Awolowo University Teaching Hospital Complex, (OAUTHC) and Seventh-day Adventist hospital after an informed oral consent and necessary ethical approvals obtained with protocol number ERC/2015/02/03under registration numbers (International: 0004553, National: NHREC/27/02/2009a).

Sample analysis and identification: The samples were inoculated directly into sterile tryptone soy broth (TSB) and incubated at 37°C for 18-24 h. Subsequently, overnight cultures from the TSB were inoculated on cetrimide agar plates supplemented with glycerol 10 mL L^{-1} and re- incubated at 37°C for 18-24 h. Primary identification of *Pseudomonas aeruginosa* isolates was based on the appearance of the colonies on cetrimide agar. The identity of the isolates was further confirmed by conventional biochemical tests.

Antibiotic susceptibility testing: Antibiotic susceptibility of the isolates was done by the Kirby-Bauer's disk diffusion technique[10]. Standardized inoculum (adjusted to 0.5 McFarland standard) was seeded on the Mueller Hinton agar plates. The antibiotic disks; single (Oxoid UK) and combine (Abtek Biological limited, UK) include: imipenem (10 µg), cefotaxime (30 µg), piperacillin (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), ceftriazone (30 µg), cloxacillin (5 µg), amoxicillin (30 µg), tetracyclines (30 µg), nitrofurantoin (200 µg), nalidixic acid (30 µg), cotrimoxazole (25 µg), erythromycin (5 µg), augmentin (30 µg), gentamicin (10 µg) and ofloxacin (10 µg) were firmly placed on the surface of the culture plates using a sterile forceps and incubated in an inverted position at 37°C for 18-24 h. The diameter of zones of inhibition was measured using a calibrated ruler to the nearest millimeter. The zone of inhibition was compared with Clinical and Laboratory Standard Institute (CLSI)[11], interpretative chart.

DNA extraction: The DNA of the isolates was extracted by suspending bacterial colonies in 200 µL of sterile distilled water in well labeled Eppendorf tubes. Tubes were covered and sealed with paraffin tape to prevent accidental opening of the tubes. The bacterial suspension was boiled at 100°C for 7 min in water bath and cold shocked in ice for 2 min. They were centrifuged at 10,000 rpm for 45 sec; the supernatant contained the DNA and was used for the polymerase chain reaction (PCR).

Table 1: Primers used for the amplification of resistance genes

Primers	Sequence 5[1]-3[1]	Gene	Amplicon size (bp)
CTX-M F	CGATGTGCAGTACCAGTAA	bla_{CTX-M}	585
CTX-M R	TTAGTGACCAGAATAAGCGG		
TEM-1 F	CCCCGAAGAACGTTTTC	bla_{TEM-1}	516
TEM-1 R	ATCAGCAATAAACCAGC		
SHV-1 F	AGGATTGACTGCCTTTTTG	bla_{SHV-1}	392
SHV-1 R	ATTTGCTGATTTCGCTCG		

Source: Colom *et al.*[12]

Polymerase chain reaction: Twenty-five micro liter of total mixture containing 4 µL of 10X buffer (Fermentas), 0.5 µL $MgCl_2$, 3 µL dNTPs (Fermentas) and 0.2 µL Taq polymerase (Fermentas), 1 µL of forward and 1 µL of reverse primers (Fermentas) and 5 µL of template DNA was set up in PCR vial. The PCR vial was placed in PCR machine (PRIME, UK) and it was subjected to initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min. A final extension procedure was carried out at 72°C for 5 min. The primers used for amplification are shown in Table 1.

Electrophoresis: Polymerase chain reaction (PCR) products were resolved on 1% agarose gel prepared by dissolving 1 g of agarose powder (Promega, Madison, USA) in 100 mL of 1X Tris-borate-EDTA (TBE) buffer (Promega, Madison, USA) solution inside a clean conical flask. The 1% agarose solution was heated in a microwave oven for 2-3 min and was observed for clarity which is an indication of complete dissolution. The mixture was then allowed to cool to about 50°C after which 0.5 µL of ethidium bromide (Promega, Madison, USA) was then added. The 10 µL mixture was loaded to the well of agarose gel electrophoresis. Power supply was adjusted to 100 Volts for 25 min and gel was placed into the 1X TBE buffer in the tank. For each run, a 100 base-pair molecular weight DNA standard (100 bp size marker) was used to verify the proper size for each PCR product and the photograph was taken.

Statistical analysis: Data were analyzed and presented using frequency distribution, mean±SD and percentages.

RESULTS

A total of thirty-one adult patients were recruited within the period of September, 2014 and June, 2015. The patients included 11 (35.5%) females and 20 (64.5%) males (Table 2) and the mean age (±SD) was 62.2 (±25.8). As shown in Table 3, the prevalence of *Pseudomonas aeruginosa* only was 16.2%, while mixed culture of *Pseudomonas aeruginosa* with other organisms was 22.6%. Meanwhile 29% of the samples yielded no organisms on the cetrimide agar.

Table 2: Baseline characteristics of the patients with chronic respiratory diseases

Characteristics	Cases number (%)
Mean age±SD	62.2±25.8
Sex	
Female	11 (35.5)
Male	20 (64.5)
Marital status	
Single	4 (12.9)
Married	27 (87.1)
Patients status	
Outpatients	22 (71.0)
Inpatients	29 (29.0)
Religion	
Christianity	24 (77.4)
Islamic	7 (22.6)
Occupation	
Civil/public servants	5 (16.1)
Professional/managers	1 (3.2)
Farming/artisans/trading	13 (41.9)
Pensioners	3 (9.7)
Dependant/unemployed	6 (19.4)
Others	3 (9.7)
Occupational exposure	
Yes	18 (58.1)
No	13 (41.9)
Cough duration	
<3 months	3 (9.7)
3 months and above	28 (90.3)

Antimicrobial resistance phenotypes of *Pseudomonas aeruginosa*: Resistance to antibiotics varied among the isolates (Table 4). High resistance profile was observed against most antibiotics namely augmentin, piperacillin, cloxacillin among others. However, the organisms were most sensitive to ceftazidime and gentamicin (8.3%).

Multiple antibiotic resistance (MAR) phenotypes of the *Pseudomonas aeruginosa*: All the isolates showed multiple antibiotic resistance patterns (Table 5). About 3(25%) of the isolates were the highest with multiple antibiotic resistant pattern to 7 classes of antibiotics, while 1(8.3%) isolate showed the least with multiple antibiotic resistant pattern to 5 classes of antibiotics tested.

Resistant genes profile of the *Pseudomonas aeruginosa*: Figure 1a shows the agarose gel electrophoresis of bla_{CTX-M} (585 bp) in the organisms. The isolates harboured bla_{SHV-1} (392 bp) (Fig. 1b) and bla_{TEM-1} (516 bp) genes as represented in Fig. 2a and b.

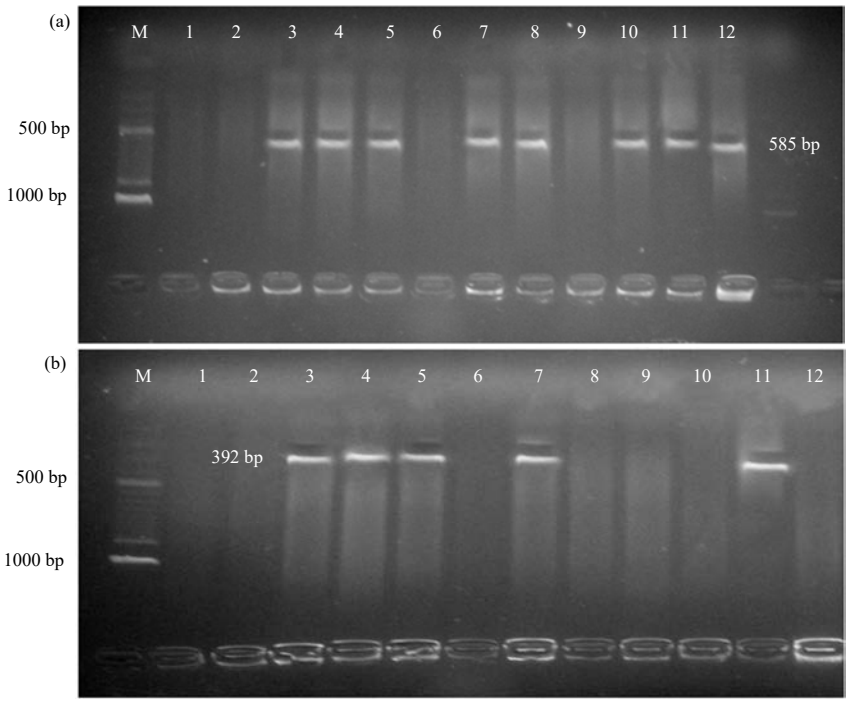

Fig. 1(a-b): Agarose gel electrophoresis of (a) *bla*$_{CTX-M}$ gene in *Pseudomonas aeruginosa*, (*bla*$_{CTX-M}$ amplicon size 585 bp) and (b) *bla*$_{SHV-1}$ gene in *Pseudomonas aeruginosa*, (*bla*$_{SHV-1}$ amplicon size 392 bp)

Table 3: Occurrence of *Pseudomonas aeruginosa* isolated from the sputum samples of recruited patients

	Frequency of isolation (%)					
	Outpatients (n = 23)		Inpatients (n = 8)		Total (n = 31)	
Etiologic agents	Number	Percentage	Number	Percentage	Number	Percentage
P. aeruginosa only	4	12.9	1	3.2	5	16.2
P. aeruginosa with other organisms	5	16.2	2	6.5	7	22.6
Other organisms only	6	19.4	4	12.9	10	32.3
No organism isolated	8	25.8	1	3.2	9	29.0

Table 4: Antibiotic resistance profile of the *Pseudomonas aeruginosa*

	Total (n = 12)	
Antibiotics	Number	Percentage
Imipenem	2	16.7
Ceftazidime	1	8.3
Cefotaxime	6	50.0
Cefuroxime	12	100.0
Ceftriazone	9	75.0
Ofloxacin	4	33.3
Gentamicin	1	8.3
Augmentin	12	100.0
Piperacillin	12	100.0
Cloxacillin	12	100.0
Tetracyclines	12	100.0
Amoxicillin	12	100.0
Nitrofurantoin	12	100.0
Nalidixic acid	11	91.7
Cotrimoxazole	12	100.0
Erythromycin	12	100.0

Table 5: Multiple antibiotic resistance (MAR) phenotypes of the *Pseudomonas aeruginosa*

Classes of antibiotics tested	Multiple antibiotics resistance pattern	Frequency	Overall	
			Number	Percentage
5	AMX, COT, ERY, NIT, TET	1	1	8.3
6	AMX, COT, ERY, NAL, NIT, TET	4	8	66.7
	COT, CTR, ERY, NAL, NIT, TET	2		
	COT, CTX, ERY, NIT, OFL, TET	2		
7	AMX, COT, ERY, IMP, OFL, NIT, TET	1	3	25.0
	COT, CRX, ERY, IMP, NAL, NIT, TET	1		
	CAZ, COT, GEN, ERY, NIT, OFL, TET	1		

Table 6: Prevalence of beta-lactamase genes among the *Pseudomonas aeruginosa* isolates from patients with chronic respiratory diseases

Beta-lactamase genes	Isolate source					
	Inpatient's		Outpatient's		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
bla_{CTX-M}	3	25.0	5	41.7	8	66.7
bla_{TEM-1}	1	8.3	4	33.3	5	41.7
bla_{SHV-1}	2	16.7	3	25.0	5	41.7

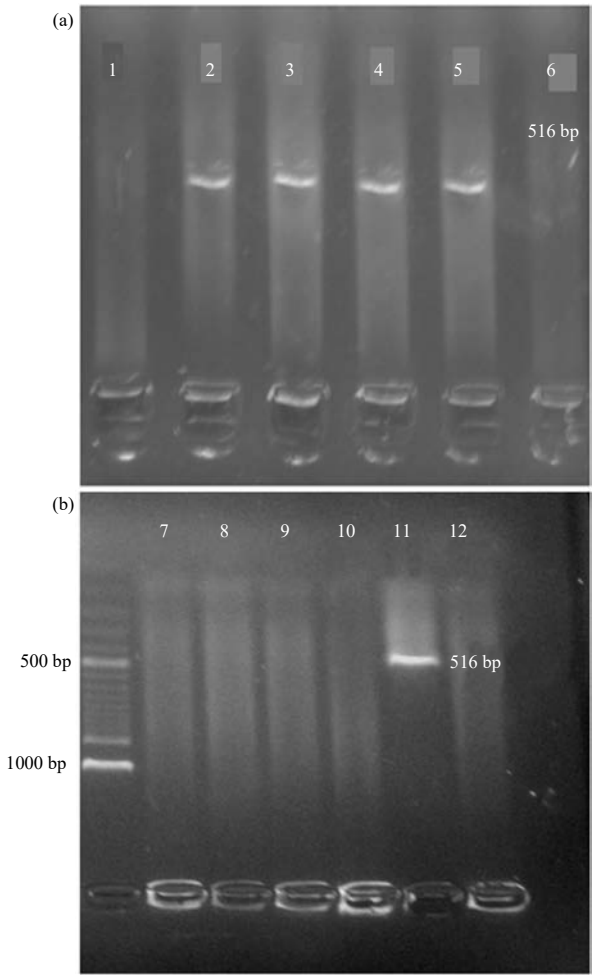

Fig. 2(a-b): Agarose gel electrophoresis of (a) *bla*TEM-1 and (b) bla_{TEM-1} gene in *Pseudomonas aeruginosa*, (*bla*TEM-1 and bla_{TEM-1} amplicon size 516 bp

The prevalence of beta-lactamase genes among the *Pseudomonas aeruginosa* isolates from in and out-patients is presented in Table 6. The bla_{CTX-M} (585 bp) was the predominant gene harboured by the organisms (66.7%).

DISCUSSION

Isolation of *Pseudomonas aeruginosa* from patients with chronic respiratory diseases is a risk factor for increased exacerbations of chronic respiratory infections[13-18], experienced by some of these patients[4]. The prevalence of *Pseudomonas aeruginosa* observed in chronic respiratory diseases in the present study was 38.8%.This is significantly higher than previous findings by other authors reporting 16.0[4], 5.1[15] and 16.5%[19]. Similarly, a prevalence of 9.7% *Pseudomonas aeruginosa* was reported from bronchiectasis patients based on a two-year prospective study[20]. However, the prevalence of *Pseudomonas aeruginosa* observed in the present study was similar to the 35% reported, in patients with COPD[3]. Furthermore, a prevalence of 20% *Pseudomonas aeruginosa* from COPD patients[21] and 24% *Pseudomonas aeruginosa* from lower respiratory tract infections have been reported by Fatima *et al.*[22].

Pseudomonas aeruginosa is widely known for its high rate of resistance to antibiotics and has been severally reported for this unique ability[23-26]. Strikingly, this feature was observed in this present study, as all the *Pseudomonas aeruginosa* isolates from the patients were resistant to multiple antibiotics. This is a worrisome development as several studies have linked multidrug resistance to increased morbidity and mortality[27-30]. Resistance

to multiple classes of antibiotics severely limits therapeutic options available for treatment of infections in patients with chronic respiratory diseases. This may further increase the morbidity and mortality. It was however observed that ceftazidime, gentamicin, imipenem and ofloxacin were the most sensitive antibiotics against the *Pseudomonas aeruginosa* isolates which is consistent with earlier reports by Fatima *et al.*[22], Qi *et al.*[31], Forozsh *et al.*[32], Mohanasoundaram[33] and Naqvi *et al.*[34], this might be due to the low usage of these agents. As shown in this study, *Pseudomonas aeruginosa* isolates were all resistant to commonly used and available drugs like augmentin, erythromycin and others.

The acquisition of the highly resistant *Pseudomonas aeruginosa* by the patients may be due to several factors, overuse/misuse of antibiotics[13], previous history of hospitalizations[16] and age[35] as seen in this study. Most of the patients recruited were 60 years and above, also the low rate of isolation of organisms among inpatients may be a reflection of multiple antibiotic usages. The study confirmed a genetic basis for resistance in most of the isolates with the detection of beta lactamase genes (bla_{TEM-1}, bla_{SHV-1}, bla_{CTX-M}). The beta lactamase genes were responsible for resistance against most or all antibiotics used in the study[36]. However, other mechanisms of resistance might have been employed by the organisms against the drugs[37], but these were not established during this study.

This study is limited because of small sample number and inability to identify other organisms and their sensitivity profile. However, despite this, it has provided useful information for further studies.

CONCLUSION

Chronic respiratory diseases were mostly prevalent in ages 60 years and above. More out-patients compared to in-patients with chronic respiratory diseases, had *Pseudomonas aeruginosa* infection. There is significant presence of β-lactamase resistance genes in the isolates responsible for multi drug resistance. The isolates were mostly sensitive to ceftazidime, gentamicin, imipenem and ofloxacin. There is therefore an urgent need for better antimicrobial stewardship for treatment of chronic respiratory infections.

SIGNIFICANCE STATEMENT

This study discovered the presence of beta lactamase genes (bla_{TEM-1}, bla_{SHV-1} and bla_{CTX-M}) in the isolates which predisposed them to high rate of resistance to most antibiotics as observed in the study. However, the study confirms that the isolates were significantly sensitive to ceftazidime, gentamicin, imipenem and ofloxacin. This therefore presents with limited therapeutic options which need to be administered cautiously.

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