



Molecular Characterization of Multidrug-resistant Bacteria Isolated From Patients With Pneumonia at Two Hospitals in North-West Nigeria

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Abstract

Background and aims: The spread of antimicrobial resistance (AMR) is a serious public health threat complicating treatment and resulting in prolonged hospitalization. The prevalence of AMR threat is not well defined due to the dearth of appropriate surveillance systems. This study sought to assess the prevalence of AMR among bacterial isolates from sputum specimens obtained from patients with pneumonia presenting at two secondary healthcare facilities in Zaria from June 1 to August 31, 2018.

Methods: Standard methodology was followed in processing sputum samples that met the acceptance criteria. The antibiotic susceptibility patterns of bacterial pathogens cultured from sputum specimens obtained from June 1 to August 31, 2018) were evaluated using the recommendation of the Clinical and Laboratory Standards Institute. Finally, data were analyzed using descriptive statistics.

Results: *Acinetobacter* spp. were the predominant pathogens accounting for 32% of recovered isolates, followed by *Staphylococcus* spp. (18%) and *Klebsiella* spp. (17%), respectively. AMR was found in 91% of the isolates. Most isolates were resistant to erythromycin (ERY) (80%) and amoxicillin (83.3%). Eventually, the multiple antibiotic resistance index ≥ 0.3 was observed in 76% of the isolates.

Conclusion: Based on the findings, AMR rates were observed to be high, and may display a serious therapeutic challenge to the management of community-acquired pneumonia. Concerted efforts are needed to combat the worrisome AMR trends revealed in this study.

Keywords: *Acinetobacter* spp., Antibiotic resistance, Community-acquired pneumonia, *Klebsiella* spp.

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Introduction

Of respiratory tract infections, pneumonia remains a major cause of morbidity and mortality in both children and adults, particularly in low and middle-income countries.¹ It kills an estimated 1 million children under the age of 5 every year and accounts for 16% of deaths in preschool children² with around 90% occurring in the developing world^{3,4} and low-income countries, where access to medical care is often difficult and the availability of routine vaccination is still below global standards.⁵ According to Liu et al, of the 6.3 million reported deaths in children in the first 5 years of life worldwide in 2013, 52% died of infectious disease, and pneumonia has been responsible for about 15% of the total deaths.⁶

In an Australian review of pneumonia in adults aged ≥ 65 years in 2012, about 78 000 general practitioner visits due to pneumonia were recorded during 2008-2013, and nearly 43 336 pneumonia hospitalizations were along reported in 2011-2012.⁷ Pneumonia is a global public health threat and most severe cases have been reported to be of bacteria-associated type.⁸ It has been suggested that

reduced immune function as a result of comorbidities,⁹ previous but resolved viral respiratory infection, and high microbial load during colonization may be predisposing factors to bacterial pneumonia.¹⁰⁻¹² *Streptococcus pneumoniae*, *Haemophilus influenzae* have been reported as predominant bacterial pathogens associated with pneumonia.¹³ Gram-negative bacilli (e.g., *Klebsiella* spp., *Acinetobacter* spp.) have been demonstrated as the emerging threat.¹⁴ Antibiotic resistance threatens the limited armory of drugs available for treating common infections, a trend which is expected to continue.¹⁵ Empiric antibiotic therapy for pneumonia has consisted of beta-lactams, especially amoxicillin (AML) for non-complicated cases, injectable cephalosporins (e.g., ceftriaxone, CRO) for severe cases, and combinations which could be a macrolide (erythromycin, ERY), aminoglycoside (gentamicin, GEN), or a respiratory fluoroquinolone.^{16,17} However, penicillin-resistant *S. pneumoniae*, ampicillin-resistant *H. influenzae*, methicillin-resistant *Staphylococcus aureus* (MRSA, both community- and hospital-acquired),¹⁸ and multidrug-resistant (MDR) Enterobacteriaceae have been

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implicated in pneumonia cases.

Antimicrobial resistance (AMR) increases drug costs and the length of stay and adversely affects the patient's outcome. Resistance to all classes of antibiotics has developed to various extents among the common and important pathogens. The most frequently reported mechanism of resistance to beta-lactams (common among gram-negative bacteria) is the production of penicillin hydrolyzing enzymes, called beta-lactamases. The presence of beta-lactamases dates back to the pre-antibiotic era, and was first reported just over a decade after the discovery of penicillin.¹⁹ The first beta-lactamase, TEM, named after a Grecian patient, Temoneira, was first reported in 1963,²⁰ and then, the sulfhydryl variable (SHV) beta-lactamase was reported ten years later, which are able to hydrolyze penicillins and narrow-spectrum cephalosporins.²¹

The extended-spectrum beta-lactamases (ESBLs), which were capable of hydrolyzing penicillin and cephalosporins (especially expanded spectrum cephalosporins), were identified in 1980.²² These are believed to have originated from the TEM-1, TEM-2, and SHV-1 enzymes, and differ from their parents by single amino acid substitutions.²³

The introduction of beta-lactamase inhibitors to counteract the effect of these enzymes in the 1980s did work out for a while.²⁴ However, new variants of the enzymes that are not affected by the activity of these beta-lactamase inhibitors abound, and range from the complex mutant of TEM (CMT) which hydrolyze both 3rd generation cephalosporins and possess poor affinity for clavulanic acid, to the metallo-beta-lactamases which are not affected by both avibactam and clavulanic acid.²⁵ Currently, there are thousands of identified beta-lactamases, and new discoveries as research into these compounds have a continuous trend.

The other mechanisms of resistance to beta-lactams are the modification of the penicillin-binding proteins (serine acyltransferases that catalyze the formation of cross-linked peptidoglycan and the target of β -lactam antibiotics) usually reported in Gram-positive and some Gram-negative organisms.²⁶ The most commonly reported mechanism of macrolide resistance is that mediated by ERY ribosomal methylase (*erm*) genes. These genes coordinate the modification of the ribosome of host bacteria, hence, ERY is unable to bind. They are achieved by the addition of two methyl residues to a highly conserved adenine residue in domain V, the peptidyl transferase center of 23S rRNA, leading to a conformational change in the ribosome.²⁷

Several variants of these genes have so far been identified in this regard. The *ermA* gene, commonly found among Staphylococci spp., the *ermB* gene is reported in both Gram-negative and -positive organisms while the *ermC* gene is detected among organisms.²⁸

Another mechanism of macrolide resistance is the efflux pump mediated by the *msr* and *mef* genes, which code for low-level resistance,²⁹ however, high-level macrolide

resistance is expressed in combination with *erm* genes. Macrolide-inactivating phosphotransferases are coded for by *mph*, which are arranged in tandem and expressed from the same promoter as the macrolide efflux pump.³⁰

Therefore, the aim of this study is to determine the frequency of microbial agents and their antibiotic resistance patterns in pneumonia over a 3-month period (June 1 to August 31, 2018) in Zaria, Nigeria.

Materials and Methods

This study was performed by focusing on two hospitals in Zaria metropolis viz Hajiya Gambo Sawaba hospital, and Kofan-gaya, Zaria, Kaduna State, Nigeria. This is a 200-bed capacity facility with over 5000 admissions annually and is located within Zaria, the Zaria Local Government area while Saint Luke's Anglican Hospital is located in Wusasa, Kaduna State, Nigeria, and is a private secondary health care provider with over 3000 admissions annually. Patients of all age groups presenting at the hospital and diagnosed as having pneumonia by a physician. Ninety patients with community-acquired pneumonia presenting at the two care facilities were included in the study.

Sample Collection, Processing and Identification of Organisms

The collected sputum specimens from June 1 to August 31, 2018 were processed using standard methods. Blood, MacConkey, and Mannitol salt agars (Titan Biotech Ltd, India) were used for the isolation of bacteria. The samples were inoculated onto prepared agar plates, which were incubated at 37°C for 24 hours in an aerobic atmosphere. Standard biochemical tests using Microgen ID kits (Microgen Bioproducts Ltd, UK) were employed to identify the organisms.

Antibiotic Susceptibility Testing

The bacteria were tested against a panel of antibiotics using the guidelines of the Clinical Laboratory Standards Institute,³¹ and the tested antibiotics were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England) and included GEN (30 µg), streptomycin (S, 30 µg), and amoxicillin-clavulanate (AMC, 30 µg). Other antibiotics were AML (30 µg), vancomycin (VAN, 30 µg), oxacillin (OXA, 10 µg), CRO (30 µg), ceftazidime (CAZ, 30 µg), cefoxitin (FOX, 30 µg), ciprofloxacin (CIP, 5 µg), and trimethoprim-sulfamethoxazole (SXT, 1.25 µg + 23.75 µg). In addition, ERY (15 µg), azithromycin (AZT, 15 µg), linezolid (LZD, 10 µg), quinupristin-dalfopristin (QD, 15 µg), tetracycline (TET, 30 µg), and imipenem (IMP, 10 µg) were other employed antibiotics. The modified Kirby-Bauer disc-diffusion method was used to determine the antibiotic susceptibility of isolates identified and confirmed by biochemical tests. An overnight culture of each isolate was prepared in nutrient agar and incubated at 37°C for 18 hours. Five milliliters (5 mL)

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of sterile physiological saline and 0.5 McFarland turbidity standard solutions were prepared for the standardization of inoculums. The discrete colonies of isolates on nutrient agar plates were emulsified in 5 mL of sterile physiological saline and the turbidity adjusted to 0.5 McFarland standard (approximately a cell density of 1.5×10^8 cfu/mL). The standardized suspension was inoculated on Muller-Hinton agar at an angle 60° across the plate using a sterile cotton swab to ensure even distribution and confluent growth. The plates were allowed to dry for 5 minutes. The disc of various antibiotics was aseptically placed using a sterile forceps on the dried inoculated agar surface. After 30 minutes of applying the discs to allow for pre-diffusion, the plates were incubated at 37°C for 18 hours. After incubation, the plates were examined for the zones of inhibition and result interpretation according to the Clinical and Laboratory Standards Institute (CLSI).³¹ The multiple antibiotic resistance index (MARI), which is an indicator of the level of exposure to antibiotics, was determined as well.^{32,33}

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which resistant}}{\text{Total number of antibiotics tested}}$$

For the purpose of this study, non-susceptibility to one or more antibiotic(s) in at least three classes was considered as multidrug resistance.

Statistical Analysis

IBM Statistical Package for Social Sciences (Version 22, International Business Machines Corporation) was used for data analysis, and the results are presented as frequencies, percentages, and means.

Results

The gender distribution of diagnosed pneumonia cases is provided in Table 1. A total of 49 males (54.4%) and 41 females (45.6%) were sampled within the period, giving a male-female ratio of 1.2. The highest and the lowest number of samples were collected from 16-49 and 6-15 age groups, respectively. No samples were collected from children aged <5 .

Of the ninety collected sputum specimens, seventy-eight were positive for bacterial growth. Gram-negative bacteria made up more than 70% of the recovered isolates (Table 2). *Acinetobacter* spp. [*A. baumannii* (06), *A. Iwoffii* (16) and *A. haemolyticus* (03)], *Staphylococcus* spp., and *Klebsiella* spp. [*K. pneumoniae* (09), *K. ornithinolytica* (02), *K. oxytoca* (01), and *K. ozaenae* (02)] were the predominant isolates.

Based on the results (Table 3), most Gram-negative isolates exhibited resistance to AML and ERY. *Acinetobacter* spp. showed high resistance to ERY (65%), AMC (45%), and TMP/SMX (35%).

Klebsiella spp. were resistant to ERY (100%), AML

Table 1. Gender and Age Distribution of Diagnosed Pneumonia Cases

Gender	Ages (y)			Total (%)
	6-15	16-49	≥ 50	
Male	5	31	13	49 (54.4)
Female	2	30	9	41 (45.6)
Total (%)	7 (7.8)	61 (67.8)	22 (24.4)	90 (100)

Table 2. Distribution of Isolated Bacteria

Bacteria	(% Isolates) (n=78)
<i>Staphylococcus</i> spp.	18
<i>Streptococcus</i> spp.	5
<i>Acinetobacter</i> spp.	32
<i>Klebsiella</i> spp.	17
<i>Enterobacter</i> spp.	9
<i>Serratia</i> spp.	6
<i>Hafnia alvei</i>	1
<i>Proteus mirabilis</i>	1
Other Enterobacteriaceae	10
Unspecified	1
Total	100

(92.3%), CRO (77%), FOX (30%), TMP/SMX (30%), and TET (23%). One *Klebsiella* isolate demonstrated resistance to IMP. *Serratia* spp. were resistant to ERY (100%), AML (100%), ceftazidime (100%), FOX (100%), TET (60%), and TMP/SMX (60%). *Enterobacter gergoviae* represented high resistance to ERY (100%), AML (100%), CRO (50%), FOX (67%), TET (50%), and TMP/SMX (50%). The results also revealed that *Hafnia alvei* was resistant to the tested antibiotics including IMP. CIP, IMP, and GEN each showed broad activity against most gram-negative isolates.

Of the gram-positive isolates (i.e., *Streptococcus* spp.) demonstrated no resistance to any of tested antibiotics. Coagulase positive *Staphylococcus* spp. indicated high resistance to ERY (78%), LZD (78%), quinupristin-dalfopristin (QD, 78%), AML (78%), oxacillin (56%), TET (44%), TMP/SMX (44%), S (33%), and CIP (22%). Coagulase negative *Staphylococcus* spp. showed high resistance to ERY (100%), QD (100%), AML (100%), oxacillin (100%), TET (100%), TMP/SMX (100%), S (100%), and CIP (100%). Further, 50% of the *Staphylococcus* spp. isolates were methicillin resistant.

Based on the findings, 91% of the isolates were resistant to at least one antibiotic with multidrug resistance observed in 47% of the isolates while only 9.1% of them were susceptible to all tested antibiotics. The percentage of multidrug resistance shown by the isolates is presented in Table 4.

Table 5 provides the MARI of bacterial isolates. In this study, 75.8% of organisms indicated an MARI of greater than or equal to 0.3. Ten MDR isolates were investigated

Table 3. Antibiotic Resistance Pattern of Bacterial Isolates

Bacteria Isolate	Antibiotics (% Resistant)											
	ERY	CIP	CN	TET	SXT	S	AML	FOX	LZD	QD	AZM	OX
Gram-positive												
<i>Staphylococcus</i> spp. (n= 12)	66.7	25	16.7	50	50	41.7	75	50	58.3	66.7	16.7	91.7
<i>Streptococcus</i> spp. (n= 2)	0	0	0	-	-	0	0	-	0	-	0	-
Gram-negative												
	IMP	ERY	CIP	CN	TET	SXT	AMC	S	AML	CAZ	CRO	FOX
<i>Acinetobacter</i> spp. (n= 20)	0	65	0	0	5	35	45	30	5	-	-	-
<i>Klebsiella</i> spp. (n=13)	7.7	100	0	0	23	30.1	-	7.7	92.3	0	76.9	30.1
<i>Enterobacter</i> spp. (n=6)	0	100	16.7	0	50	50	-	33.3	100	0	50	66.7
<i>Serratia</i> spp. (n=5)	0	100	0	0	60	60	-	40	100	100	-	100
<i>Hafnia alvei</i> (n=1)	100	100	100	100	100	100	100	100	-	-	-	-
<i>Proteus mirabilis</i> (n=1)	0	100	0	0	100	100	-	100	100	0	-	100
Other Enterobacteriaceae (n=6)	0	83.3	0	16.7	33.3	50	-	66.7	-	-	-	-

Key: - = not tested.

CN: Gentamicin; CIP: Ciprofloxacin; CRO: Ceftriaxone; SXT: Trimethoprim-sulfamethoxazole; IMP: Imipenem; AML: Amoxicillin; AMC: Amoxicillin-Clavulanate; S: Streptomycin; TET: Tetracycline; CAZ: Ceftazidime; ERY: Erythromycin; FOX: Ceftazidime; AZM: Azithromycin; QD: Quinupristin-dalfopristin; LZD: Linezolid; OX: Oxacillin.

Table 4. Percentage of Multidrug Resistance Among Tested Bacterial Isolates

Bacteria	(% isolates) MDR
<i>Staphylococcus</i> spp. (n= 12)	83.3
<i>Streptococcus</i> spp. (n= 2)	0
<i>Acinetobacter</i> spp. (n= 20)	30
<i>Klebsiella</i> spp. (n= 13)	92.3
<i>Enterobacter</i> spp. (n= 6)	83.3
<i>Serratia</i> spp. (n= 5)	100
<i>Hafnia alvei</i> (n= 1)	100
<i>Proteus mirabilis</i> (n= 1)	100
Other Enterobacteriaceae (n= 6)	50

Note. MDR: Multidrug-resistant.

for the carriage of resistance genes. The molecular detection of the *ESBL* and *ermB* genes revealed that 7 isolates were positive for the *TEM* gene, with 5 harbouring the *ermB* gene while 3 of them were positive for *OXA* (Figure 1 and Table 6, respectively).

Discussion

Pneumonia ranks high among the killer infectious diseases of children and adults, affecting both low- and high-resource countries. Mortality rates are kept at the minimum by a combination of early diagnosis, appropriate treatment protocols, and the diligent management of associated comorbidities and risk factors.^{34,35}

In this study, Gram-negative bacilli were the predominant isolates, which is in line with reports in south-western Nigeria^{36,37} and Cambodia.³⁸ Microbial communities are known to vary with the geographic location. In this study, the majority of the recovered ones were Gram-negative bacilli, which corroborates with reports in Indonesia³⁹ and Bangladesh.⁴⁰ Gram-positive cocci, however, was reported

Table 5. MARI of Bacterial Isolates From Patients With Pneumonia in Zaria, Nigeria

MARI	Percent
0.0	9.1
0.1	10.6
0.2	4.5
0.3	26.0
0.4	12.1
0.5	15.1
0.6	10.6
0.7	3.0
0.8	3.0
0.9	1.5
1.0	4.5
Total	100%

Note. MARI: Multiple antibiotic resistance index.

as being predominant in Bosnia⁴¹ and Sweden.⁴²

Bacteria-associated pneumonia, which accounts for the most severe cases, is complicated by the threat of antibiotic resistance, which is also found in our study. The observed resistance levels to AML (45-100%) and ERY (65-100%) were worrisome, considering that beta-lactams, particularly AML, are generally adopted as first-line drugs in empirical treatment, occasionally in combination with macrolides (e.g., ERY).⁴³

The prevalence of MDR isolates (47%) was high in this study, which could be due to over-exposure to and the possible misuse of antibiotics. El-Sokkary et al⁴⁴ reported higher MDR rates in a similar study in Egypt. *Staphylococcus* spp., *Acinetobacter* spp., and *Klebsiella* spp. accounted for most of the MDR isolates in this study, which is consistent with the reports of extensively drug-resistant *Acinetobacter*

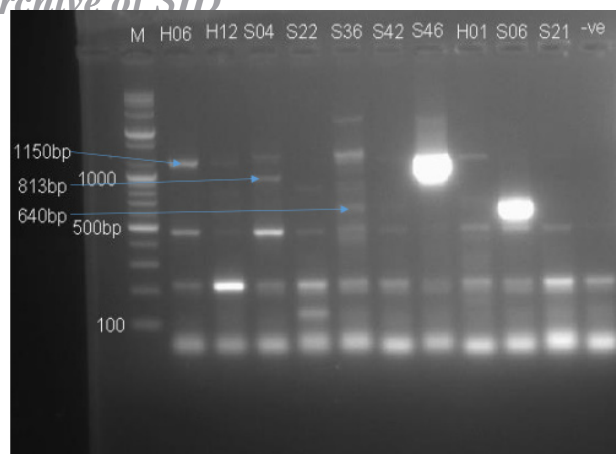


Figure 1. Electrophoregram of *TEM* (1150 bp), *OXA* (813 bp), and *ermB* (640 bp) Genes Amplified From Bacteria Isolates. Note: TEM: Transmission electron microscopy; OXA: Oxacillin. Key: M=1.5 kb ladder, -ve=Negative control. H01, H06, H12, S04, S06, S21, S22, S36, S42, S46=Bacteria isolate codes.

spp. isolated from pneumonia patients.⁴⁵ Observed methicillin resistance in *Staphylococcus* spp. was higher compared to reports of 33.3% from Ethiopia.⁴⁶ MRS spp. have previously been implicated in nosocomial cases although a recent report revealed community-acquired – MRS pneumonia, particularly CA-MRSA.⁴⁷ The low resistance of *Staphylococcus* species to GEN and CIP in this study indicated that these could be a useful alternative for treatment in cases where first-line antibiotics represent a failure. An Ethiopian study reported similar findings.⁴⁷ There was no resistance to any of the tested antibiotics in *Streptococcus* spp., which is contrary to the results of studies from Egypt and Ethiopia^{44,47} regarding resistance.

Most Gram-negative isolates were resistant to ERY and AML although GEN, CIP, and IMP could be alternatives for MDR bacteria-associated pneumonia cases within these settings since there was less resistance. El-Sokkary et al⁴⁴ reported similar effectiveness of carbapenems and fluoroquinolones in Egypt although it contrasts with the significant resistance to amikacin and CIP reported in India.⁴⁸

The presence of carbapenem-resistant Enterobacteriaceae in this study is of public health importance because the treatment of infections caused by these organisms is extremely difficult.⁴⁹ Carbapenem-resistant Enterobacteriaceae in pneumonia patients has been reported as well.⁵⁰ Resistance to IMP is low in these setting, as observed in this study, probably due to controlled prescription and high costs of these antibiotics making them inaccessible to over-the-counter self-medication, the possibility of transfer of carbapenem-resistant genes to susceptible bacteria remains, and could be deleterious

The high level of antibiotic exposure in the community could be responsible for the high observed MARI. A similar figure was reported in children with suspected septicaemia presenting at the Institute of Child Health, Zaria.⁵¹ The

Table 6. Detection of *TEM*, *OXA*, and *ermB* Genes in MDR Bacterial Isolates

Isolates	Detected Gene		
	<i>TEM</i> (1150 bp)	<i>OXA</i> (813 bp)	<i>ermB</i> (639 bp)
H01	+	-	+
H06	+	-	-
H12	+	-	+
S04	+	-	-
S06	-	-	+
S21	-	-	-
S22	-	+	-
S36	+	+	+
S42	+	-	+
S46	+	+	-

Note: TEM: Temoneira; OXA: Oxacillin; MDR: Multidrug-resistant.

observed resistance rates in this study are worrisome, and the possibility of the transfer of resistance determinants to drug-susceptible bacteria presents a looming danger. Policies for reducing the development and spread of AMR in bacteria pathogens include antimicrobial stewardship, discouraging misuse, overuse and indiscriminate over-the-counter antibiotic prescriptions, and encouraging patients' medication compliance.

The code of molecular resistance determinants for specific antibiotic non-susceptibilities in some cases contributes to cross-resistance between antibiotics within the same or different class thus limiting treatment options.⁵² The detection of *TEM-1* and *OXA-1* genes in seven (7) and three (3) of the tested (10) isolates suggests possible inclinations with encountered AML and CRO resistance in the selected isolates, respectively. The detection of *OXA* and *TEM* genes in two (2) of the isolates suggests the presence of multiple resistance mechanisms. *TEM* and *OXA* genes are known to mediate beta-lactam resistance through the production of enzymes that inactivate the antibiotic.^{53,54} Beta-lactams are generally classified into narrow and broad spectrums although these gene-mediated enzymes have evolved producing variants which act on them making them ineffective.²⁵ Infections caused by organisms possessing these genes can increase the length of hospital stay and result in intensive care unit admission. Early detection is important because the inappropriate therapy of these complex infections can increase mortality and morbidity.

The presence of *ermB* genes conferring resistance to macrolides is of importance to physicians' prescription patterns.⁵⁵ Treatment protocols may need to be reassessed to forestall community or hospital epidemic of treatment failures due to antibiotic resistance. To the best of our knowledge, there were no available reports on the molecular characterization of *ESBLs* and ERY ribosomal methylase (*ermB*) genes from the sputum of patients with

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pneumonia in Nigeria at the time of compilation of this report. However, a similar study in Japan reported the isolation of *ESBL* genes from the sputum of pneumonia patients.⁵⁶ Another report of the isolation of *OXA* type and *TEM* genes in the *Acinetobacter baumannii* isolate from the sputum of the neonatal pneumonia patient was found in China.⁵⁷

The presence of *TEM*, *OXA*, and *ermB* genes pose a significant threat to the current antibiotic therapy for pneumonia since this could culminate in treatment failures leading to a prolonged hospital stay and fatal outcomes.

Conclusion

The results of this study showed that AMR rates were observed to be high and may display a serious therapeutic challenge to the management of community-acquired pneumonia. Thus, concerted efforts are needed to combat the worrisome AMR trends revealed in this study.

Conflict of Interest Disclosures

The authors declared no conflict of interests.

Ethical Approval

Ethical approval was obtained from the Ethics Committee of the Health and Research of the Kaduna State Ministry of Health (MOH/ADM/744/VOL.1/462).

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