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Identification of Multiple Drug-Resistance Genes in Clinical Isolates of *Acinetobacter baumannii*

Michael, G.[1, 2], Garba L.[2]*, Adamu M.T.[2]., Saleh Z.M[2]. Isa S.[2] and Manga, M.M.[1,3]

[1]Department of Medical Microbiology and Immunology, Federal Teaching Hospital Gombe, POB 0037, Off Ashaka Road, Gombe State, Nigeria.
[2]Department of Microbiology, Faculty of Science, Gombe State University, PMB 127, Tudun Wada Gombe, Gombe State, Nigeria.
[3]College of Medical Sciences, Gombe State University, PMB 127, Tudun Wada Gombe, Gombe State, Nigeria.

*Corresponding author:
Garba Lawal,
Department of Microbiology,
Faculty of Science,
Gombe State University,
P.M.B. 127, Tudun Wada Gombe,
Gombe State,
Nigeria.
Email: lagarpak@gmail.com

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ABSTRACT

Around the world, *Acinetobacter baumannii* is a nosocomial pathogen that accounts for 80% of hospitalized patient infections. This opportunistic bacterium can cause a wide range of infections, including skin and soft tissue infections, meningitis, pneumonia acquired on a ventilator, urinary tract infections, and bacteremia, all of which have a high death rate of almost 63.3%. Finding multidrug-resistant genes in the clinical isolates of *A. baumannii* was the goal of this study. Twenty-four (24) *A. baumannii* clinical isolates were obtained from the Federal Teaching Hospital in Gombe, Nigeria, and subcultured on MacConkey agar for eighteen to twenty-four hours at 37 ˚C. The isolates were identified using traditional biochemical testing, morphology, and cultural traits. The VITEK was then used to validate the identification. Twenty of the 24 isolates had been identified as *A. baumannii*. The Kirby Bauer method and CLSI recommendations were utilized to determine the antibiotic susceptibility of the *A. baumannii* isolates. Polymerase Chain Reaction was used to identify the genes that were resistant to many drugs. According to the data, fifteen (15) *A. baummanii* isolates, or 75% of the total, were likely multidrug resistant. The presence of the BlaNDM-1 gene in eight isolates, the BlaOXA23 gene in six isolates, and the BlaVIM gene in just four isolates was confirmed by molecular detection of the MDR genes from these isolates. These results show that a significant portion of the isolates of *A. baumannii* carried genes resistant to many drugs, which could be the root cause of any infection treatment failure caused by this organism.

INTRODUCTION

Multidrug-resistance has been on the rise in nosocomial infections linked to *Acinetobacter baumannii* in recent decades. According to Kostyanev et al. [1], *A. baumannii* is a nosocomial pathogen that is fast gaining popularity. It can cause serious infections such as meningitis, pneumonia, bacteremia, urinary tract infections, and wound infections. Due to its extraordinary tendency to quickly acquire resistance determinants to a wide range of antibacterial drugs, it has now emerged as a major global cause of hospital-acquired infections. *A. baumannii* was added to the World Health Organization's priority pathogen list for investigation and development of novel antibiotics, according to the Lancet 2022 publication on drug-resistant diseases [2].
Numerous nosocomial infections are caused by multidrug-resistant bacteria [3]. Many investigations into the prevalence and molecular characterization of multidrug-resistant *Acinetobacter baumannii* have been carried out in various locations, including Tahiti in the South Pacific [4], Lagos, Zaria, Kano, and Owerri in Nigeria [5-7], and Saudi Arabia [8]. According to Uwingabiye et al. (2016)[9], the rates of Acinetobacter infections were 19.2% in Asia, 17.1% in Eastern Europe, 14.8% in Africa, 13.8% in Central and South America, 5.6% in Western Europe, 4.4% in Oceania, and 3.7% in North America. In Nigeria, Osogbo recorded 8.5% [10]; Owerri, in Southwest Nigeria, recorded 9.0% [7]. Global health catastrophe brought on by multidrug-resistant (MDR) strains causing persistent outbreaks in hospitals [11-12]. *A. baumannii* has always been sensitive to various antibiotics. However, the widespread multidrug resistance phenotype has made it resistant to fluoroquinolones, β-lactams, aminoglycosides, and even colistin [13].

In their study on the molecular characterization of an *Acinetobacter baumannii* outbreak, Al-Hassan and Al-Madboly [14] described multidrug resistant *A. baumannii* (MDR *A. baumannii*) as a problematic hospital pathogen with limited therapeutic options that may lead to a high morbidity and mortality rate [15]. The Infectious Disease Society of America included MDR *A baumannii* on its ESKAPE infectious organism list because of its extensive distribution in hospitals [14, 16]. Multidrug resistant microorganisms have presented new challenges to humanity in the modern era [17].

Antibiotic resistance, according to Wall [18], can harm people worldwide without taking into account demography. According to estimates, infectious diseases that can be treated with therapeutic medicines claim the lives of 5.7 million people annually, the majority of whom reside in low- and middle-income countries (LMICs) [19]. The Ad hoc Interagency Coordinating Group on Antimicrobial Resistance of the United Nations (UN) estimates that by 2050, diseases brought on by drug-resistant microorganisms might claim 10 million lives annually. Treatment of common illnesses may be seriously jeopardized by the emergence of multidrug resistant pathogens such as *Acinetobacter baummanii*, *Pseudomonas aeruginosa*, and various Enterobacteriaceae members [13].

A. baumannii's development of multidrug resistance to the majority of antibiotics now used to treat severe infections is extremely concerning since it can resist treatment, including carbapenems, which are the preferred medicines for treating severe infections. The primary mechanism of resistance found in *A. baumannii* isolates is the production of β-lactamases, which include carbapenem-hydrolyzing class D β-lactamases (CHDLs), also known as oxacillinases (OXAs), as found in OXA-23, OXA-24, and OXA-58; extended spectrum-β-lactamases (ESBLs) as TEM-92, SHV-5, CTX-M-2, and CTX-M-15; and metallo-β-lactamases (MBLs) as IMP-1, VIM-1, and NDM-1 [20].

Molecular characterization offers more information on the various strains linked to nosocomial infections and the resistance genes responsible for antibiotic resistance. Phenotypic identification and antimicrobial susceptibility pattern have accounted for over 90% of research conducted on Acinetobacter species in Nigeria's tertiary hospitals [6]. Thus, the purpose of this study was to identify MDR genes in isolates of *Acinetobacter baumannii* from different samples that were brought to the Federal Teaching Hospital's laboratory in Gombe.

MATERIALS AND METHODS

Ethical approval
The Federal Teaching Hospital in Gombe, Nigeria's Research and Ethics Committee awarded ethical approval with reference number NHREC/25/10/2013.

Clinical isolates' source
Twenty-four (24) clinical isolates of *Acinetobacter* species were gathered from the Federal Teaching Hospital, Gombe's Medical Microbiology/Immunology laboratory.

Confirmatory tests of isolates
Following the manufacturer's instructions, blood agar and MacConkey agar plates (Sigma-Aldrich) were produced, and Acinetobacter species were grown on these media for 18 to 24 hours at 37 ºC. After the incubation period of one night, the isolates of Acinetobacter species were verified through Gram staining and biochemical tests, which included the VITEK 2 system as instructed by the manufacturer (BioMérieux), the Catalase test, the Citrate utilization test, the Indole test, the Oxidase test, the Motility test, the Triple Sugar Iron (TSI) agar test, and the Urease test [21-24].

Phenotypic detection of multidrug-resistant *Acinetobacter bauamannii*
Acinetobacter baumannii isolates were subjected to phenotypic testing against various antibiotic classes (Oxoid Laboratories). These antibiotic classes included ampicillin-sulbactam (10/10 µg), ceftazidime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), meropenem (10 µg), piperacillin-tazobactam (100/10 µg), ceftriazone (30 µg), tipecycline (10 µg), cefepime (30 µg), tetracycline (30 µg), and amikacin (30 µg). *Acinetobacter baumannii* colonies were cultivated on Mueller Hinton Agar plates for 24 hours at 37 ˚C, followed by inoculum standardization, before being submitted to sensitivity.

Inoculum standardization
Colonies of *A. baumannii* were removed from the overnight growth culture using a sterile wire loop, and they were then dissolved in 4 milliliters of sterile physiological saline. The suspension's turbidity was measured in relation to the 0.5 McFarland Standard [23].

Antibiotic sensitivity test
Using the modified Kirby-Bauer disk diffusion method [23] and guidelines from the Clinical and Laboratory Standard Institute in Wayne, USA [25], the susceptibility of the *A. baumannii* isolates was ascertained. The standardized inoculum of every *A. baumannii* isolate was applied to Mueller Hinton agar plates that had been prepared using a sterile swab stick. The agar surface was allowed to dry on the inoculation plates for 3-5 minutes at room temperature.

The individual antibiotic discs were placed on the inoculation plates using sterile forceps, about 15 mm from the plate edge. The plates were then incubated for 24 hours at 35 °C. The growth inhibition zone diameter was measured in millimetres and interpreted in accordance with CLSI guidelines [25]. Until further examination, isolates that tested positive for at least three antibiotic classes were kept in 20% glycerol stock at -20 °C.

Molecular detection of multidrug-resistance genes
In Kano State, Nigeria, at the Advanced Biomedical and Public Health Initiative's molecular laboratory, molecular detection was carried out.

Genomic DNA extraction
Ten (10) mL of nutrient broth was inoculated with a single colony of *A. baumannii* that had been grown overnight on a plate, and then incubated for twenty-four hours at 37 °C. Following the manufacturer's instructions, Quick-DNA Mini Prep plus kits (Zymo, Research, Germany) were used to extract the genomic DNA of the bacterium cultivated in the broth culture. The DNA was analysed on a 1% (w/v) agarose gel.

PCR Detection of multidrug-resistance genes
In a 12.5-µL total PCR reaction mixture containing 6.25 µL master mix (Dream Taq Green PCR Master mix, Thermo Scientific), 4.25 µL sterile distilled water, 0.5 µL of each forward and reverse primers (Table 1), and 1 µL DNA template, three (3) distinct multidrug-resistant genes were successfully detected by PCR.

Table 1. Primers sequences adopted for amplification of the target genes.

Primer	Sequence	Ref
Bla NDM-1- F	(GAT GGT GTT TGG TCG CAT A)	[26]
Bla NDM-1- R	(CGA ATG CGC AGC ACC AG)	
blaOXA-23-F	(5′GATCGGATTGGAGAACCAGA3′)	[27]
blaOXA-23-R	(5′ATTTCTGACCGCATTTCCAT3′)	
blaVIM- F	(5′TTGACCGCGTCATCATGGC3′)	[20]
blaVIM- R	(5′ACCGCCTGCTCTAATGTAAG3′)	

Using different incubation settings, PCR amplifications were carried out on a thermocycler (Senso-Quest Labcycler, Germany) to identify the resistance genes (Table 2). 1% (w/v) agarose gel was used to analyze the PCR products, and Cleaver Scientific's Omnidoc gel documentation system was used to visualize the results.

Table 2. PCR Amplification conditions.

Genes	1st denaturation	Denaturation	Annealing	Extension	Final extension
BlaNDM-1	1 cycle	35 cycles			1 cycle
	94°C/ 5min	94°C/ 45s	60°C/45 s	72°C/30s	72°C/ 10min
blaOXA23	1 cycle	30 cycles			
	94°C/ 5min	94°C/ 60s	50°C/60s	72°C/90s	72°C/ 10min
blaVIM	1 cycle	30 cycles			
	95°C/ 5min	95°C/ 30s	52.5°C/30s	72°C/30s	72°C/ 8min

RESULTS

The study was carried out to detect the presence of MDR genes from the clinical isolates of *Acinetobacter baumannii* obtained from Federal Teaching Hospital, Gombe. The isolates were confirmed and subjected to different antibiotics for phenotypic determination of multidrug- resistance. The antibiotic resistance genes were detected from the isolates using conventional PCR amplification.

Confirmation of *Acinetobacter* species

Following the overnight incubation of all isolates on blood agar base and MacConkey agar, cultural characteristics showed colonies to be non-lactose fermenting organisms. The isolates were confirmed as Gram-negative, motile, citrate positive, urease negative, oxidase negative, non-glucose or sucrose fermenting that do not produce hydrogen sulphide or gas. The biochemical tests confirmed 24 isolates to be Acinetobacter species. Furthermore, the 24 isolates were subjected to VITEK 2 COMPACT system of identification, which differentiated them into three different species of Acinetobacter comprising *Acinetobacter baumannii*, *Acinetobacter iwoffii* and *Acinetobacter baumannii* calcoaceticus complex (ACB). The *A. baumannii* was found to be the most occurring with 20 isolates corresponding to 83.3%, followed by *A. iwoffii* with 2(8.3%) isolates and then *Acinetobacter baumannii* calcoaceticus complex (ACB) with 2(8.3%) isolates (Table 3).

Table 3. Percentage occurrence of *Acinetobacter baumannii*.

Species	Occurrence	Percentage (%)
A. baumannii	20	83.3
A. iwoffii	2	8.3
A. baumannii calcoaceticus complex(*ACB)*	2	8.3
Total	24	100

Phenotypic detection of multidrug-resistant *Acinetobacter baumannii*

Antibiotic susceptibility test conducted according to Kirby Bauer method and in line with CLSI guidelines showed that, out of the 20 isolates of *Acinetobacter baumannii*, fifteen (15) isolates corresponding to 75.0% were multidrug-resistant (MDR). Specifically, 93.3% of the *A. baumannii* were resistant to ceftriaxone and cefepime, 86.7% to ceftazidime and tigecycline, 80.0% to each of levofloxacin, ampicillin/sulbactam and tetracycline, 73.3% to each of piperacillin/tazobactam and amikacin, 66.7% to ciprofloxacin while meropenem and gentamicin each recorded resistance of 60.0% (**Fig. 1**).

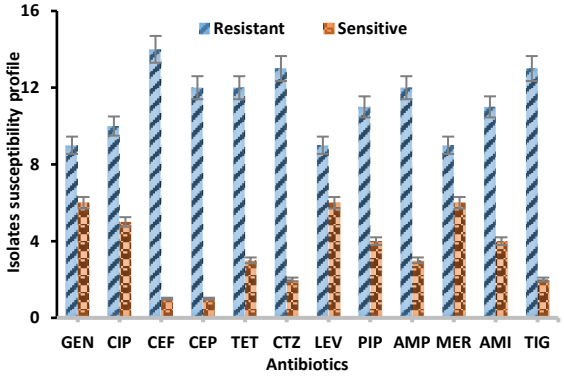

Fig. 1. Antibiotic susceptibility pattern of *Acinetobacter baumannii*.

Molecular detection of Multidrug-resistant *A. baumannii*

The PCR products analyzed on 1% (w/v) agarose gel showed the presence of approximately 390 bp BlaNDM-1 gene in isolates 1, 2, 6,7,8,9, 13 and 14 (**Fig. 2**). BlaOXA23 gene was amplified from isolates 2,6,7,8, 13 and 14 (**Fig. 3**) while BlaVIM gene was only amplified from isolates 2, 8, 14 and 15 (**Fig. 4**).

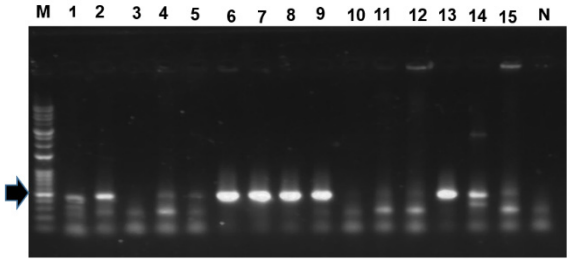

Fig. 2. PCR product of blaNDM-1 amplified from *A. baumannii* isolates on 1% (w/v) agarose gel. M= 1kb DNA ladder (250-10,000bp), 1-15: *A. baumannii* isolates, N: Negative control. The expected size of the gene is 390 bp indicated an arrowhead.

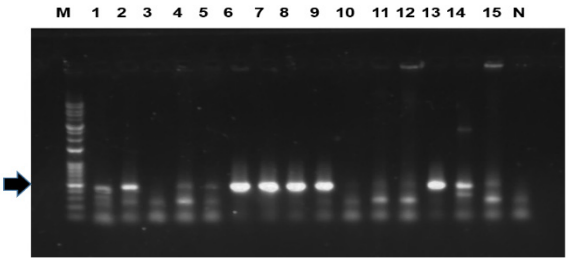

Fig. 3. PCR products of blaOXA amplified from *A. baumannii* isolates on 1% (w/v) agarose gel. M= 1kb DNA ladder (250-10,000 bp), 1-15: *A. baumannii* samples, N: Negative control. The expected size of the gene is 501 bp indicated by the arrow head.

DISCUSSION

One of the ESKAPE pathogens linked to hospital acquired illnesses, *A. baumannii* is also a major contributor to outbreaks related to healthcare [6]. Furthermore, this organism is now a global multidrug-resistant microbe that poses a severe threat to

medicine because it is resistant to several last-resort treatments [21,28]. It is important to remember that *A. baumannii* was linked to important clinical samples in the study that was done, and that most laboratory studies may have missed it.

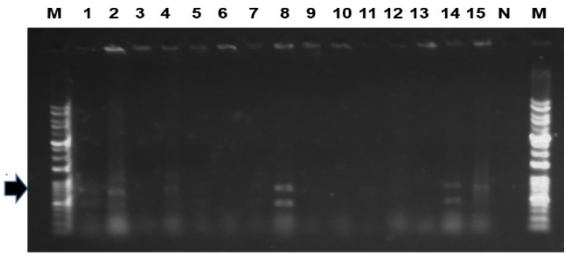

Fig. 4. PCR products of bla VIM amplified from *A. baumannii* isolates on 1% (w/v) agarose gel. M= 1kb DNA ladder (250-10,000 bp), 1-15: *A. baumannii* isolates, N: Negative control. The expected size of the gene is 762 bp indicated by the arrow head.

Of the twenty-four Acinetobacter isolates that were obtained, fifteen (62.5%) came from surgical wards, three (12.5%) from out-patient departments (OPD), and two (8.3%) from medical wards; the remaining isolates came from the ICU, ANC/O&G, pediatric wards, and orthopaedic wards, each with one (~4.2%) isolate. This may be because surgical wound sites are vulnerable to Acinetobacter species infections if they are not well managed. Acinetobacter species is a known cause of nosocomial infections. *A. baumannii* were found in the following wards according to a similar study by Rynga et al. [29]: ICU (28%), burns ward and respiratory medicine ward (15% each), surgical ward (14%), burns ICU (11%), gynecology ward (9%), orthopedics ward (5%), and respiratory medicine out-patient department (2%).

With a 99.9% confidence level, the VITEK 2 COMPACT system confirmed that, of the 24 Acinetobacter species, 20 (7.1%) were *Acinetobacter baumannii*, 2 (0.7%) were *Acinetobacter iwoffii*, and 2 (0.7%) were *Acinetobacter calcoaceticus*. Numerous investigations have determined that the automated VITEK 2 COMPACT System is helpful, accurate, and produces results quickly when it comes to identifying and testing for antibiotic susceptibility in general and *A. baumannii* in particular [21,28,30].

The majority of the medications used to treat bacterial infections were ineffective against the isolates of *A. baumannii*. According to Magiorakos et al. [31], bacteria that exhibit resistance to three (3) distinct antibiotic classes are categorized as multidrug-resistant. In cases where the isolates exhibit both MDR and carbapenem resistance, the term "extensive drug resistance" is employed. As a result, fifteen (15) of the twenty isolates of *A. baumannii* in this study were multidrug-resistant (MDR). Contrary to the results of this investigation, Pal et al. [32] stated that *A. baumannii* exhibited high resistance to cephalosporin, whilst Odewale [10] reported that the organism exhibited 100% resistance to ciprofloxacin and amikacin. This could be as a result of the widespread and simple access to these antibiotics.

The presence of the multidrug-resistant genes bla OXA (40%), bla NDM-1 (66.7%), and bla VIM (26.7%) in the *A. baumannii* isolates was verified by molecular detection. Ten isolates containing the blaNDM-1 gene were found to contain roughly 66.7% of the MDR genes. This is consistent with other findings on the global establishment and dissemination of *A. baumannii*, which harbors NDM-1 [1]]. Additionally, it has been revealed that the blaNDM-1 gene and the blaOXA-23 gene are related [1]. Similar findings were seen in this investigation, where 40% of the isolates were discovered to carry both the blaOXA-23 and blaNDM-1 genes. In a study carried out in Saudi Arabia, Selim et al. [33] found that the *A. baumannii* isolates had a greater prevalence of the blaNDM-1 gene (8.5%). However, no indication of blaNDM genes in *A. baumannii* was found in a different investigation carried out in China by Shi et al. in 2021 [27]. This may indicate regional differences in the global prevalence of blaNDM-1. blaNDM-1 carrying *A. baumannii* has been identified as a chimeric gene created by the fusion of the aminoglycoside-resistance gene aphA6 with a mannose-binding lectin gene in numerous countries, including Germany, Spain, Israel, Egypt, Switzerland, and Libya, India, Pakistan, and Nepal [34]. Furthermore, A. *baumannii* can conjugate with its receivers to transfer the blaNDM-1 genes to them [34].

The second carbapenemase-coding gene, blaOXA-23, was found in two-thirds of the isolates. Six (40%) of the MDR *A. baumannii* isolates had this gene. This is marginally less than the results of Kostyanev et al. [1] and Al-Tamimi et al. [21], which demonstrated that 67.7% and 98.5%, respectively, of the *A. baumannii* isolates carried the blaOXA-23 gene. According to Joshi et al. [34], *A. baumannii* mostly produces carbapenemases of the OXA type. The second prominent genetic determinant in this study is the acquired bla Oxa-23, which is the dominant genetic determinant in Asia. According to reports, the blaOXA gene is found on a plasmid and can be conjugated between *A. baumannii* to spread antibiotic resistance in bacteria worldwide [34].

In this investigation, 4 isolates (26.7%) had the BlaVIM gene. In a similar vein, the findings of Al-Tamimi et al. [21] and Selim et al. [33] were 26.6% and 25.7%, respectively. One study conducted across healthcare settings in Africa detailed the intercontinental spread of *A. baumannii*, particularly strains harboring the bla_OXA-58 and NDM-1 genes. This spread is facilitated by international patient transfers, underscoring the global challenge of controlling *A. baumannii*. Notably, the OXA-23-producing strain was implicated in multiple infection outbreaks across several African countries, indicating a widespread issue with carbapenem-resistant *A. baumannii* in the region [35].

CONCLUSION

To conclude, fifteen (15) MDR *Acinetobacter baumannii* strains were found. Three genes—the BlaNDM-1 gene from eight isolates, the BlaOXA23 gene from six isolates, and the BlaVIM gene from four isolates were found. The results indicate that a significant proportion of *A. baumannii* strains include genes resistant to multiple drugs.

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