

Molecular detection of bla_{OXA-23} gene and bla_{OXA-51} gene in carbapenem resistant strains of *Acinetobacter baumannii* in patients with ventilator associated pneumonia at tertiary care hospitals

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Abstract

Objective: To evaluate carbapenem resistance and to detect bla_{OXA-23} and bla_{OXA-51} genes in carbapenem-resistant *Acinetobacter baumannii* isolates recovered from patients having pneumonia secondary to ventilation.

Methods: The cross-sectional study was conducted from July 2017 to June 2018 at the Department of Microbiology, University of Health Sciences, Lahore, Pakistan, and comprised endotracheal aspirates / tracheobroncheal lavage samples from patients irrespective of age and gender who developed pneumonia after being on the ventilator for 48 hrs at the Combined Military Hospital, and Jinnah Hospital, Lahore. The samples were inoculated on MacConkey and blood agar and aerobically incubated at a temperature of 37°C for 18-24 hours. The isolated organisms were further assessed by standard morphological, cultural and biochemical profile. Antibiotic susceptibility was done by Kirby-Bauer disc diffusion method. Carbapenem-resistant *Acinetobacter baumannii* were checked for carbapenemase production using Modified Hodge Test. Conventional polymerase chain reaction and agarose gel electrophoresis were performed to detect bla_{OXA-23} and bla_{OXA-51} genes. Data was analysed using SPSS 17.

Results: Out of 157 samples, 92(58.6%) yielded growth of bacteria, and, among them, 39(42.4%) were identified as *Acinetobacter baumannii*. All (100%) *Acinetobacter baumannii* cases showed resistance to carbapenem, were producing carbapenemase enzyme, and were positive for bla_{OXA-51} gene. The bla_{OXA-23} gene was amplified in 38(97.4%) isolates.

Conclusion: Bla_{OXA-23} gene appeared to be the major cause of carbapenem resistance.

Keywords: Ventilator-associated pneumonia, Carbapenem, *Acinetobacter baumannii*. (JPMA 71: 2576; 2021)

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Introduction

Ventilator-associated pneumonia (VAP) is the inflammation of the lung parenchyma secondary to microbial infections that happens in individuals who have required mechanical ventilation through an endotracheal or tracheostomy tube for at least 48 hours. VAP occurs in patients who have long stay in the intensive care unit (ICU) and have high mortality rates reaching up to 28.5-44.5%.¹ Individuals who are on mechanical ventilation are mostly unconscious and are not able to communicate. The most common findings are fever or hypothermia, production of purulent sputum, and reduced oxygen saturation in the blood.² *Pseudomonas* (P.) *aeruginosa*, *Klebsiella* (K.) *pneumoniae*, *Acinetobacter* (A.), *Serratiamarcescens*, *Enterobacter* (E.), *Stenotrophomonas maltophilia*, methicillin-resistant *staphylococcus aureus* (MRSA) and *Citrobacter* (C.) are the important causes of VAP.

An estimated 8% to 28% of individuals on ventilator support due to any cause are prone to develop VAP.^{3,4} VAP due to *Acinetobacter* is becoming one of the leading

causes of death (7-40%).⁵ Patients usually withstand their primary disease, but it is the lung infection due to *Acinetobacter* that is lethal. Giving prompt treatment against this organism is a very important aspect to preventing the emergence of multidrug resistant (MDR) *acinetobacter*. Therefore, it is the basis of choosing the right antibiotics against the resistant strains of *A. baumannii* and at the right time for the critically ill patients admitted in ICUs. *Acinetobacter* is a gram-negative, non-fermentative cocco-bacilli. It is oxidase-negative, catalase-positive and is a non-motile, non-fastidious aerobic organism. About 33 species are found so far.^{6,7} *Acinetobacter* is a natural environmental organism which is recovered mostly from dirt or soil as well as from the human skin.⁸ Due to increase in the number of difficult-to-treat diseases caused by *A. baumannii*, it is of great concern for physicians and microbiologists to treat the infections efficiently and promptly.⁹

Carbapenems remain the drug of choice to treat resistant strains of *Acinetobacter* because of their wider spectrum of anti-bacterial activity and fewer side-effects. But unfortunately because of their injudicious and excessive usage, MDR strains of *Acinetobacter* have emerged, thus presenting intrinsic and MDR determinants. However, resistance to carbapenems alone is sufficient to name

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A. baumannii as a highly resistant organism. In *A. baumannii*, the cause of this resistance is the emergence and production of carbapenem-hydrolyzing class D β -lactamases (oxacillinases; CHDLs) and to some extent metallo β -lactamases (MBLs). In *Acinetobacter*, these CHDLs could be intrinsic ($\text{bla}_{\text{OXA-51-like}}$) or acquired ($\text{bla}_{\text{OXA-23-like}}$, OXA-24-like and OXA-58-like).

The history of emergence of the enzyme OXA-23 dates back to 1985 when in Scotland it was discovered in *A. baumannii* isolate and showed minimum inhibitory concentration (MIC) of 16 mg/L for imipenem, and then known as acute respiratory infection-1 (ARI-1).¹⁰ Further study found this enzyme to be a cause of resistance in *Acinetobacter* strains and OXA-23 was classified under Ambler class D β -lactamases.¹¹

Resistance is shown in a number of antibiotics, including amoxicillin, ticarcillin, imipenem and meropenem, due to the presence of OXA-23 β -lactamase in vivo. When changed into sensitive $\text{bla}_{\text{OXA-23}}$ negative recipient strains, the resistance level to carbapenems conferred by the enzyme is of moderate to low level, and the level increases to higher resistance with the coexistence and over-expression of efflux pump resistance-nodulation-cell division (RND)-type AdeABC. A strain having in-built OXA-23 enzyme is transferred to a recipient strain, an increased value of MIC is seen from 16-32 L-1 and the level of resistance to carbapenems again increases with the over-expression of efflux pump AdeABC, demonstrating the contribution of other hereditary variables connected with the $\text{bla}_{\text{OXA-23}}$ gene in presenting resistance.¹²

OXA-51 is intrinsic and commonly observed to be chromosomally found in *A. baumannii* and is non-transferable. It is one of the largest enzymes of the β -lactamase family, showing up to 60 enzymes.¹³ According to a study based on polymerase chain reaction (PCR) for the detection of the gene using the plasmid deoxyribonucleic acid (DNA) as template, positive results confirmed the presence of OXA-51 gene, and that the $\text{bla}_{\text{OXA-51-like}}$ gene could be recovered from these strains. Except for $\text{bla}_{\text{OXA-138}}$, nearly all the enzymes of OXA-51 enzyme group found are recognised in *A. baumannii*. OXA-51 is a diverse family of genes in which members differ by up to 16 amino acid sequences. OXA-51-like enzymes comprises many groups and subgroups are linked together by certain epidemic lineages.¹⁴

It is a known fact that bacteria undergo continuous process of genetic changes. All three studies in this regard from Pakistan¹⁵⁻¹⁷ were conducted at least five years ago, and had different aims, target populations, nature of samples and bacterial isolates. The current study was planned to

evaluate carbapenem resistance and to detect $\text{bla}_{\text{OXA-23}}$ and $\text{bla}_{\text{OXA-51}}$ genes in carbapenem-resistant *A. baumannii* isolates recovered from patients having pneumonia secondary to ventilation.

Materials and Methods

The cross-sectional study was conducted from July 2017 to June 2018 at the Department of Microbiology, University of Health Sciences (UHS), Lahore, Pakistan, and comprised endotracheal aspirates (ETAs) and tracheobroncheal lavage (TBL) samples from VAP patients irrespective of age and gender who developed pneumonia after being on the ventilator for 48 hrs at the Combined Military Hospital, and Jinnah Hospital, Lahore. The samples were inoculated on MacConkey agar and blood agar, and were incubated aerobically at 37°C for 18-24 hours. After incubation, all plates were observed for any visible growth, colonial morphology of the growth and cultural characteristics.

The identification of isolated organisms was done by standard morphological, cultural and biochemical profile (API-20NE, bioMerieux, France).

Kirby-Bauer disk diffusion method was used to test the susceptibility of the isolates to various antibiotics using commercially available antimicrobial disks (Oxoid, Basingstoke, United Kingdom) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines 2016.¹⁸ Antibiotic disks used were trimethoprim-sulfamethoxazole (1.25/23.75 μg), gentamicin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg), ampicillin-sulbactam (10/10 μg), ceftriaxone (30 μg), piperacillin (100 μg) and imipenem (10 μg), and they were incubated for 24 hrs at 37°C. The measuring of zone size and interpretation was done according to CLSI guidelines.

The Modified Hodge Test (MHT) was used for screening of *A. baumannii*-producing carbapenemase. Appearance of clover leaf-like indentation of *Escherichia* (*E.*) *coli* 25922 was seen along the growth streak of test organism in the zone of disc diffusion after 24 hours.

For the molecular detection of $\text{bla}_{\text{OXA-23}}$ and $\text{bla}_{\text{OXA-51}}$ in carbapenem-resistant *A. baumannii*, extraction of DNA was done using DNA TIANamp genomic kit while following the protocol identified by the manufacturers.

DNA amplification was performed employing commercially prepared specific primers for $\text{bla}_{\text{OXA-23}}$ and $\text{bla}_{\text{OXA-51}}$ genes (Table 1).¹⁹

Data was analysed using SPSS 17. Data was expressed as percentages and frequencies.

Table-1: Specific primers used for amplification of bla_{OXA-23} and bla_{OXA-51} gene¹⁹

S. No	Target Gene	Primers	Primer Sequences (5'-3')	Amplicon Size (bp)
1	bla _{OXA-23}	F	GATCGGATTGGAGAACCAGA	501 bp
		R	ATTCTGACCGCATTTCAT	
2	bla _{OXA-51}	F	TAATGCTTTGATCGGCCTTG	353 bp
		R	TGGATTGCACTTCATCTTG	

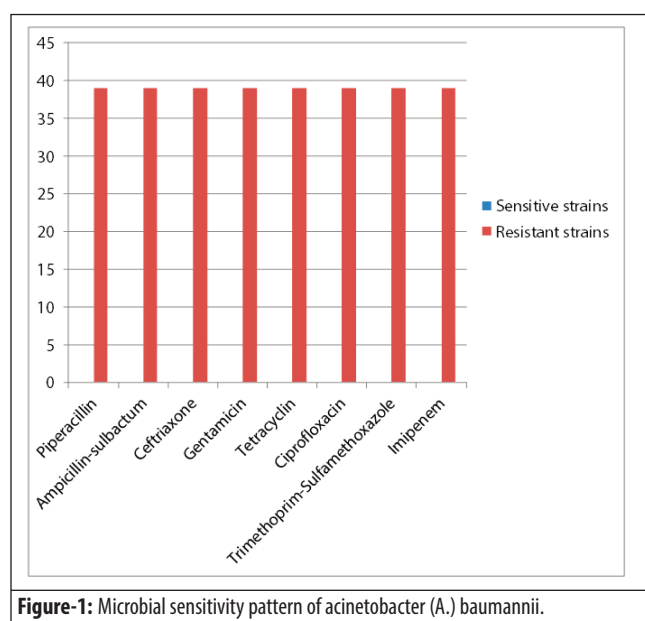
Results

Out of 157 samples, 92(58.6%) yielded growth of bacteria, and, among them, 39(42.4%) were identified as *A. baumannii*. These samples related to 27(69.2) males and 12(30.8%) females. The overall mean age was 43±13.8 years, and the largest age group was 31-40 years 11(28.2%). Of them, 36(92.3%) patients were receiving antibiotics and 3(7.7%) were not (Table 2).

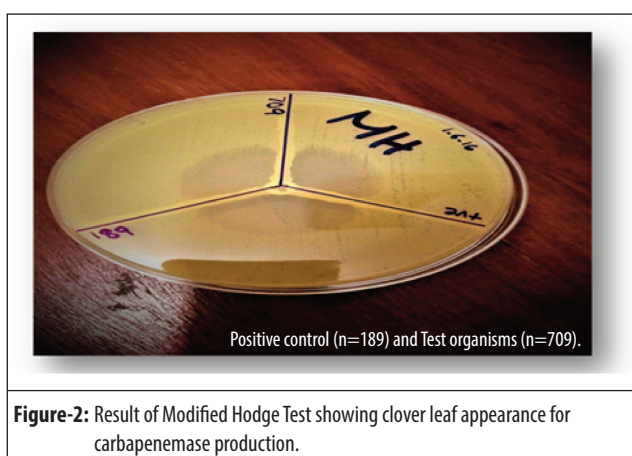
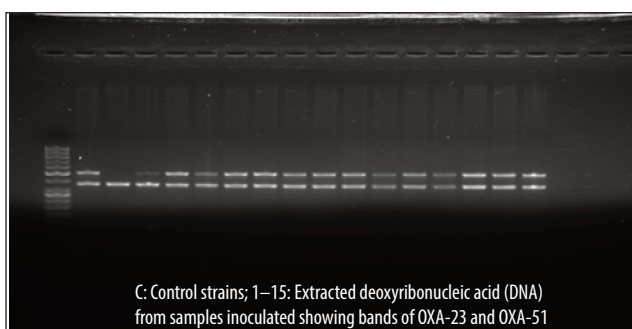
All (100%) *A. baumannii* cases showed resistance to carbapenems (Table 3; Figure 1), were producing carbapenemase enzyme (Figure 2), were positive for

Table-2: Baseline demographic characteristics of the patients (n=39).

S.No.	Characteristic	n (%)
1.	Age (years)	
	< 30	4 (10.2)
	31-40	11 (28.2)
	41-50	9 (23.1)
	51-60	8 (20.5)
	> 60	7 (17.9)
2.	Gender	
	Male	27 (69.2)
	Female	12 (30.8)
3.	Patient receiving antibiotic	
	Yes	36 (92.3)
	No	3 (7.7)

**Figure-1:** Microbial sensitivity pattern of acinetobacter (A.) baumannii.**Table-3:** Microbial sensitivity of acinetobacter (A.) baumannii (n=39).

S.No.	Antimicrobial	Sensitive strains n (%)	Resistant strains n (%)
1.	Piperacillin	0 (0)	39 (100)
2.	Ampicillin-sulbactam	0 (0)	39 (100)
3.	Ceftriaxone	0 (0)	39 (100)
4.	Gentamycin	0 (0)	39 (100)
5.	Tetracycline	0 (0)	39 (100)
6.	Ciprofloxacin	0 (0)	39 (100)
7.	Trimethoprim-Sulfamethoxazole	0 (0)	39 (100)
8.	Imipenem	0 (0)	39 (100)

**Figure-2:** Result of Modified Hodge Test showing clover leaf appearance for carbapenemase production.**Figure-3:** Detection of bla OXA-23 and blaOXA-51 genes in acinetobacter (A.) baumannii isolates. The pulsed-field gel electrophoresis (PFGE) profiles of selected clinical *A. baumannii* isolates plotted by polymerase chain reaction (PCR). The final positions and sizes (in kbp) of the molecular markers used are indicated at the top.

bla_{OXA-51} gene, while bla_{OXA-23} gene was amplified in 38(97.4%) isolates, and separated DNA fragments, or molecular markers, were present throughout the field (Figures 3-6).

Discussion

A. baumannii has been emerging as an increasing threat to mankind, causing both community and hospital-acquired infections.⁶ This notorious pathogen is one of the leading causes of VAP. *A. baumannii* is an opportunistic

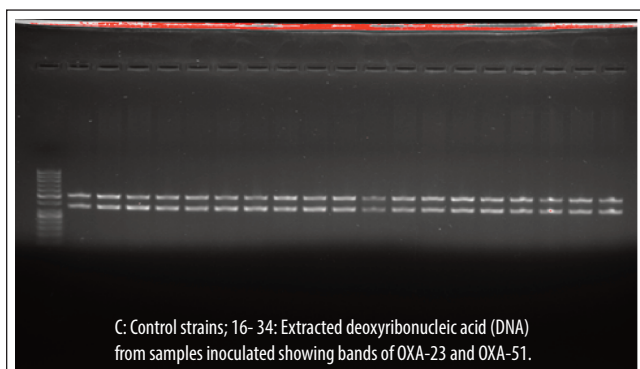


Figure-4: Detection of bla_{OXA-23} and bla_{OXA-51} genes in acinetobacter (*A.*) baumannii isolates. The pulsed-field gel electrophoresis (PFGE) profiles of selected clinical *A. baumannii* isolates plotted by polymerase chain reaction (PCR). The final positions and sizes (in kbp) of the molecular markers used are indicated at the top.

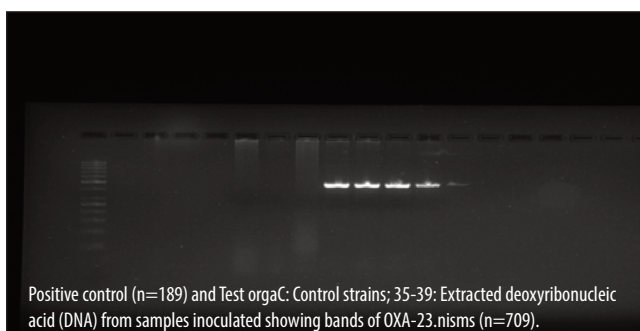


Figure-5: Detection of bla_{OXA-23} in acinetobacter (*A.*) baumannii isolates. The pulsed-field gel electrophoresis (PFGE) profiles of selected clinical *A. baumannii* isolates plotted by polymerase chain reaction (PCR). The final positions and sizes (in kbp) of the molecular markers used are indicated at the top.

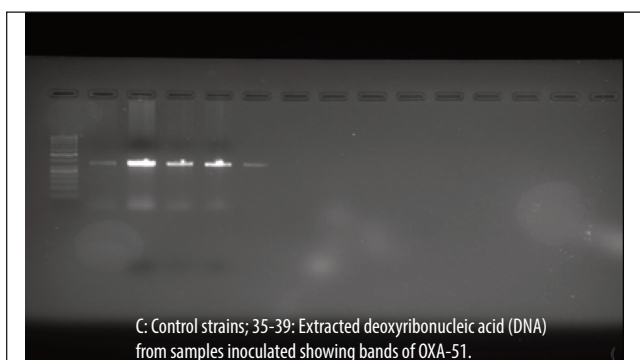


Figure-6: Detection of bla_{OXA-51} gene in acinetobacter (*A.*) baumannii isolates. The pulsed-field gel electrophoresis (PFGE) profiles of selected clinical *A. baumannii* isolates plotted by polymerase chain reaction (PCR). The final positions and sizes (in kbp) of the molecular markers used are indicated at the top.

environmental organism capable of surviving on dry surfaces as well. One of the leading sources of it is the instruments used in ICU and the infected individuals.

In the current study, all *A. baumannii* isolates were found to be resistant to carbapenems (imipenem) and were also resistant to piperacillin, ampicillin-sulbactam, ceftriaxone, gentamicin, tetracycline, ciprofloxacin and trimethoprim-sulfamethoxazole. Similar reports of MDR *A. baumannii* from healthcare facilities in China, Europe, Brazil, North America, Hong Kong, Argentina, Japan, Taiwan and Korea are part of literature.²⁰

The presence of bla_{OXA-51} in all of the samples (n=39) was expected as this very marker serves as an identification of *A. baumannii*. This finding also speaks for the diagnostic accuracy of the conventional methods of identification of bacteria.

On the other hand, bla_{OXA-23} could not be amplified in one of the 39 samples. The presence of the said marker, bla_{OXA-23}, represents the carbapenem-resistant strains of *A. baumannii*. All of the samples except one were found positive for the said marker, revealing that the sample strains which were found positive for both bla_{OXA-23} and bla_{OXA-51} were carbapenem-resistant strains of *A. baumannii*. A trial in Pakistan has reported bla_{OXA-23}-like acquired oxacillinase genes found in 47 of the total 50 isolates.¹⁶ In another study, of the 59 carbapenems resistant isolates, bla_{OXA-51}-like was common in all isolates, while bla_{OXA-23}-like in only 14 isolates.¹⁵

The absence of bla_{OXA-23} in one of the samples can be attributed to a couple of factors other than the human bias. One of the reasons might be that any mutation, no matter how localised it is (e.g point mutation), may result in change of sequence at the primer binding site(s); a substitution or deletion, for example, may result in frame shift at the primer-binding site, rendering the sequence unable to be amplified and observed. The new sequence, however, would be able enough to impart carbapenem resistance in the said strain, as is evident by the resistance pattern in the current study.

Another probable reason behind this result might be the total absence of bla_{OXA-23} in the negative sample. However, the ability of the said sample to show carbapenem resistance can be explained with reference to the origin of OXA-23-like enzyme group which has eight different enzymes known to be originated through both chromosomal and plasmid-based genomic regions. As is evident by literature, plasmids, along with other genes, harbour a great deal of drug-resistant genes, which are of great survival significance for the microorganisms.¹³ A significant motivation behind the presence of genes for 'resistance' on non-chromosomal entities might be the ease of transfer, exchange and/or modification of the genomic regions responsible for impregnating any drug and/or

other resistance in the organism harbouring it. Therefore, it might be possible that either the combination of different OXA-23-like enzymes, other than bla_{OXA-23} itself, imparts carbapenem resistance in the said negative sample strain, or there is a possibility of some other modification of drug-resistant genomic regions at the plasmid level.

Any genetically-advanced bacterium can attain and exchange genes and/or segments of the genes with another genetically compatible counterpart so that any colossal piece of mutation would not be limited to the progeny of a single colony-forming unit (CFU).⁸

Whichever might be the reason behind the presence of carbapenem resistance even in the absence of bla_{OXA-23}, it can be speculated that this very microbe is evolving right in front of us. The accomplishment of resistance to various antibiotics by different variants of *A. baumannii* is an illustration of how evolution is still employed to impart perfection to organisms in a challenging environment. Though a couple of reasons, including a great deal of mutations in the drug resistant genes and a highly sophisticated pattern of dissemination of fruitful transformations among the variants, are the most probable explanations of the said characteristics, the credit of imparting drug resistance by the adoption of non-scientific practices regarding the use of antibiotic drugs still goes to homo sapiens.

Several studies in Europe have revealed that most of the types isolated from *A. baumannii* outbreaks can be traced back to a limited number of types or heredities (clones) of *A. baumannii*.^{6,21} Similar are the findings of a study conducted in UK.²² A very few studies in Pakistan have employed molecular techniques to identify the types/clones of *A. baumannii* isolated from clinical cases. In a study,¹⁵ the majority of isolates belonged to strains of European clones I and II. However, a significant number of studies would be needed to identify the types of carbapenem-resistant *A. baumannii* clones prevalent in Pakistan, so that a phylogenetic relationship tree could be constructed to ascertain the epidemiology of the said infection. It would also help in the categorisation of genes associated with carbapenem and other drugs' resistance enzymes.

The current study would likely lead to future trials also for the comparative efficacy of conventional and molecular techniques with reference to the diagnosis of *A. baumannii*. Pre-designed, ready-to-use kits used in the current study, for instance, depicted all 39 samples positive for *A. baumannii*. Similarly, MHT revealed the presence of carbapenemase in all the positive samples. Furthermore, results of the PCR corroborated the findings of other

conventional techniques employed in the current study. It could, therefore, be suggested that techniques with limited use of time, labour and expense must be utilised, preceded by extensive trials, for diagnosis.

Conclusion

Bla_{OXA-23} gene appeared to be the major cause of carbapenem resistance. Complete DNA sequence analysis of *A. baumannii* isolated in Pakistan needs to be done in future studies for better understanding of the molecular and the genetic basis of the drug resistance. New antimicrobial drugs or novel combinations need to be developed to effectively treat this potentially lethal infection.

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Conflict of Interest: None.

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