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ASSOCIATION OF CARBAPENEMASES AND *ABAI* GENE IN BIOFILM-PRODUCING AND NON-BIOFILM PRODUCING *ACINETOBACTER BAUMANNII* CLINICAL ISOLATES TO EXPRESS CARBAPENEM RESISTANCE

Purnachandra Rao Uggirala[1], Arvind Natarajan[2*], Vijay Kumar Gangannagari[3]

[1]Ph.D scholar, Microbiology, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India.
[2]Professor, Department of Microbiology, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India.
[3]Assistant Professor, Department of Microbiology, Siddaganga Medical College and Research Institute, Tumakuru, Karnataka, India.

Abstract
Acinetobacter baumannii has become an important pathogen, with biofilm-producing strains increasingly showing resistance to multiple drugs. The rise in carbapenem resistance complicates treatment, as carbapenemases can neutralize these antibiotics. Additionally, the AbaI gene (Autoinducer synthase) is associated with quorum-sensing signals and biofilm production, contributing to drug resistance. This study aimed to explore the relationship between carbapenemases and the *abaI* gene in carbapenem-resistant *A. baumannii,* focusing on both biofilm-producing and non-biofilm-producing isolates. We collected 190 clinical isolates, dividing them into 158 biofilm producers and 32 non-biofilm producers. Among the biofilm producers, 115 were resistant to carbapenems, with 105 identified as producing both carbapenemases and the *abaI* gene. In the non-biofilm producers, 14 were carbapenem-resistant, with 12 being carbapenemase producers. Further PCR testing revealed that 105 biofilm producers were positive for the *abaI* gene and carbapenemase production, while 6 non-biofilm producers also tested positive. The results indicated a significant association between carbapenemase production and the *abaI* gene in carbapenem-resistant biofilm-producing isolates (P = 0.0001).

KEYWORDS: Biofilm, *Acinetobacter baumannii*, Carbapenem resistance.

INTRODUCTION

Acinetobacter baumannii (A.baumannii) has become an emerging pathogen in hospital-acquired infections. Its ability to form biofilm is making this organism resistant to a wide range of drugs. *A. baumannii* is the second most important pathogen, after Pseudomonas aeruginosa, to cause infections in healthcare settings. Day by day, carbapenem resistance is increasing in *A. baumannii* infections, making it more difficult to treat these cases.[1] Carbapenems are the broad-spectrum antibiotics that act against various bacteria and are a widely used class of antimicrobial drugs for the treatment of infections with multidrug-resistant A. baumannii-like pathogens. However, resistance to carbapenems has increased globally. One of the important reasons for this resistance is the production of several carbapenem-hydrolyzing enzymes called carbapenemases. In the last 2 decades, the number of newly detected carbapenemases has increased continuously.[2] Different kinds of carbapenemases (OXA-23, OXA-58, OXA-40-like), KPC, VIM, and NDM have been found in *Acinetobacter baumannii.*[3,4] Carbapenemases can be detected by CarbAcinetoNP test, which is a rapid and reproducible, phenotypic method to detect all kinds of carbapenemases with a sensitivity of 94.7% and a specificity of 93.2%. This is a cost-effective method for identifying carbapenemases in *Acinetobacter spp.*[5,] The exact virulence factors responsible for the pathogenicity of *Acinetobacter spp.* are still not well defined. Several genetically related factors and the ability to form biofilms are creating favorable conditions to cause severe infections. *abaI* gene (auto inducer synthase) is one of the virulence factors, and it codes for the synthesis of quorum-sensing molecule 3-hydroxy-C_{12}-HSL. Quorum sensing is a communication process by autoinducers, hormone-like molecules, through which bacterial cells communicate with their neighboring bacterial cells. Auto inducers act by connecting to transcriptional regulatory proteins and thus energize the gene expression in the bacterial cells. As a result of Quorum sensing, bacteria can benefit in several ways, such as cell density, virulence, motility, plasmid transfer, biofilm formation, and drug resistance.[6,7,] so the present study was taken to find out the association of carbapenemase production and *abaI* gene in biofilm-producing and non-biofilm producing clinical isolates of carbapenem-resistant *A. baumannii.*

MATERIALS AND METHODS

In this study, we collected a total of 190 previously identified isolates of *Acinetobacter baumannii* by standard conventional culture test. Among these isolates, 158 were detected as biofilm producers and 32 were non-biofilm producers, by the Tissue culture plate method. Then, carbapenem-resistant isolates among these isolates were detected by using imipenem and meropenem disks by the Kirby-Bauer disk diffusion test as per CLSI guidelines. Out of 158 biofilm-producing isolates, 115 were detected as carbapenem-resistant isolates, and out of 32 non-biofilm-producing isolates, 14

were detected as carbapenem-resistant.[8] All the carbapenem-resistant isolates from both the biofilm-producing group of strains and the non-biofilm-producing group of strains were tested for Carbapenemase detection by using the CarbAcineto NP method.[9] And the *abaI* gene detection was done by using the standard conventional PCR method.[10]

Procedure for CarbAcineto NP Method:
Reagents required:
- 5M NaCl
- Phenol red solution (o.5%)
- Zinc sulfate heptahydrate ($ZnSo_47H_2O$)
- Standard grade Imipenem powder or Injectable Imipenem-cilastatin powder**.**

Solution A: In a 25-50ml beaker, 2ml of 0.5% phenol red solution was added to 16.6ml clinical laboratory reagent water (CLRW)
- Then 180µl of 10mM $ZnSo_47H_2O$ solution was added
- PH was adjusted to 7.8 ± 0.1
- Stored at -20 °C

Solution B:
- 6mg/ml imipenem standard powder was added to solution A, and stored at -20 ^{0}C.

Controls: ATCC BAA 1705 *K.pneumoniae* for positive control
ATCC BAA 1706 *K.pneumoniae* for negative control

Protocol for CarbAcineto NP test:
- 4 pairs of Eppendorf tubes (1.5ml capacity) have been taken for test, positive control, negative control, blank, and labeled as 'A' for the first tube and 'B' for the second tube of each pair.
- 100µl of protein extraction reagent (5M NaCl) was added to each tube.
- Emulsified the colonies of isolates with a (10µl) calibrated loop from the overnight blood agar plate in both tubes A and B. Vortexed for 5 seconds. Isolates are not added in a blank
- 100µl of solution A was added to tube 'A', and solution B was added to tube 'B'. The tubes were vortexed well.
- Incubated at 35^{0}C ±2^{0}C for upto 2hrs.isolates that demonstrated the positive results before 2hrs.have been reported as carbapenemase producers.
- CarbAcinetoNP method was performed 3 times by using controls and a test strain to standardize the method.

Table 1: Interpretation of results in CarbAcineto NP Method

Tube 'A'	Tube 'B'	Result
Red or red-orange	Red or red-orange	Negative
Red or red-orange	Dark yellow or yellow	Positive
Red or red-orange	Orange	Invalid
Orange, dark yellow, or yellow	Any colour	Invalid

Conventional PCR Method for the detection of the AbaI gene:
AbaI gene (382 bp) with forward and reverse Primers.
F-5'-GTACAGTCGACGTATTTGTTGAATATTTGGG-3'
R-5'-CGTACGTCTAGAGTAATGAGTTGTTTTGCGCC-3
DNA isolation and purification:
Bacterial DNA isolation and purification from *Acinetobacter baumannii* isolates were performed as given by the DNA purification kit of Himedia Company.
Master Mix preparation for PCR test:
- PCR buffer-2.5µl
- $Mgcl_2$- 1.5µl
- dNTPS- 2.5µl
- forward primer-1µl
- reverse primer- 1µl
- Taq polymerase- 0.5µl
- Template DNA- 2.0µl
- H_2O- 14µl

PCR temperature cycling parameters:
Step 1: Initial denaturation- 94 °C for 10 minutes.
Step 2: Denaturation of 30 cycles- 94 °C for 30 seconds
Step 3: Primer annealing- 66.5 °C for 30 seconds.
Step 4: Primer extension – 720 °C for 1 minute.

Step 5: Final extension- 72 °C for 5 minutes.
The amplified products of PCR were identified by Gel electrophoresis.

Fig. PCR Gel Pic showing AbaI gene band with 382 bp

Lane 1 Ladder, Lane 2,3,4,5 are test samples, and Lane No.6 & 7 are positive control and negative controls, respectively.

RESULTS:

Table 2. Distribution of A. baumannii isolates in biofilm-producing and non-biofilm-producing groups.

Total No. of isolates N=190	Carbapenem-resistant N=129	Isolates Positive for the *abaI* gene and Carbapenemase
Biofilm producers N=158	115	105
Non-biofilm producers N=32	14	6

Statistical analysis: Chi-square test (2x2 table) and Fisher's exact test are used to find out the association between carbapenemase production and *abaI* gene in biofilm-producing and non-biofilm-producing clinical isolates of carbapenem-resistant *A. baumannii*.

Table. 3: Association of carbapenemases and *abaI* gene in biofilm-producing *A. baumannii* isolates.

Isolates	Carbapenemase-positive isolates	Carbapenemase-negative isolates	Total
abaI gene-positive isolates	105	5	110
abaI gene-negative isolates	0	5	5
Total	105	10	115 (P = 0.0001) *

P = 0.0001. Indicates an extremely significant association

Table 4. Association of carbapenemases and *abaI* gene in non-biofilm producing strains of *A.baumannii*:

A.baumannii isolates	Carbapenemase-positive isolates	Carbapenemase-negative isolates	Total
abaI gene-positive isolates	**6**	**0**	**6**
abaI gene-negative isolates	**6**	**2**	**8**
Total	**12**	**2**	**14 (P = 0.4)**

P = 0.4. Indicates no statistically significant association

DISCUSSION

Infections of *Acinetobacter baumannii* are one of the major health problems in healthcare facility centers. The biofilm-forming ability of this microbe is also influencing, like a virulence factor, and plays an important role in persistence of infection and increased drug resistance 75% of *A. baumannii* isolates can form biofilms.[11] Leu H et al. study described that biofilm-forming *A. baumanni* strains with the abaI gene are associated with a high rate of drug resistance to a wide range of drugs, including carbapenems[12]. Azizi O et al. reported that the co-existence of the carbapenemases belongs to $bla_{oxa-51,}$ $bla_{oxa-23,}$ and $bla_{oxa-24/40}$ like genes in the biofilm-forming multidrug-resistant *A.baumannii* isolates and expressed a high rate of resistance to carbapenems[13].

Zhu et al (2022), through their study, reported that all CRAB isolates had bla_{VIM} and $bla_{oxa-23.}$ Whereas Carbapenemase genes such as $bla_{oxa-51,}$ bla_{IMP}, and bla_{NDM} were present in 98.70%, 67.53%, and 31.17% of CRAB isolates, respectively[14]. Moreover, their study demonstrated that virulence genes such as abaI (quorum-sensing system) and CsuA (biofilm formation) were present in all (100%) clinical CRAB isolates, suggesting that there is a relationship between

carbapenemases and biofilm formation with the presence of the *abaI* gene. Treatment of infections caused by extensively drug-resistant *Acinetobacter baumannii* has become a big challenge in developing countries and is almost impossible for the health care systems with limited facilities.[15,16] So in the view of understanding the drug resistance mechanisms in A. baumannii, in the current study, we performed a cost effective and rapid modified carbAcineto NP method to detect carbapenemase producers, and conventional PCR method for the detection of *abaI* gene in carbapenem resistant *A. baumannii* clinical isolates, and we found that the number of carbapenemase producers with the presence of *abaI* gene is more (105) in biofilm forming carbapenem resistant *A.baumannii* isolates (115) while compared to the number of carbapenemase producers with the presence of AbaI gene (6) in non-biofilm forming carbapenem resistant *A.baumannii* isolates (14). According to these results, the present study is positively correlated with the studies of Leu H et al and, Azizi O et al., and Zhu et al. statistically, showing that there is a significant association (P value is 0.0001) of carbapenemase production and the *abaI* gene in biofilm-producing carbapenem-resistant *A.baumannii* isolates.

CONCLUSION

The findings of this study indicate a significant association between carbapenemase production and the *abaI* gene in conferring carbapenem resistance among biofilm-producing *Acinetobacter baumannii* isolates, whereas no such association was detected in non-biofilm-producing isolates.

Conflicts of interest: No conflicts of interest.

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