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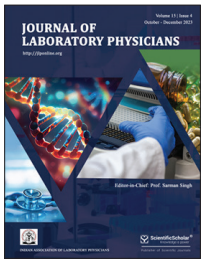

Original Article

Molecular characterization of biofilm-producing clinical isolates of carbapenem-resistant *Acinetobacter baumannii* at a tertiary care center

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ABSTRACT

Objectives: This study was aimed at identifying biofilm-producing carbapenem-resistant *Acinetobacter baumannii* (CRAB) from various clinical specimens and the possible relationship between biofilm formation and antimicrobial resistance at our clinical setup.

Materials and Methods: This observational study was conducted in the Department of Microbiology, HIMS, and Himalayan School of Biosciences at Swami Rama Himalayan University, Dehradun. A total of 72 CRAB were included and subjected to phenotypic microtiter plate assay and biofilm-forming gene detection using polymerase chain reaction.

Statistical analysis: The relationship between biofilm-forming CRAB and drug resistance was determined using a non-parametric statistical test.

Results: All 72 isolates of CRAB were uniformly found resistant to imipenem (100%) and meropenem (100%). A high level of resistance was observed against cefepime (100%), levofloxacin (99%), cotrimoxazole (70%), and minocycline (53%). Out of 72 CRAB isolates, 64 (89%) were identified phenotypically as biofilm producers and 8 (11%) as non-biofilm producers. All CRAB isolates harbored more than one biofilm-forming gene, including outer membrane protein A (ompA) (89%) and chaperon-usher pilus E (csuE) (68%). The most predominant gene, ompA, was carried by 56 (88%) biofilm producers and 8 (100%) non-biofilm producers.

Conclusions: A high frequency of biofilm-forming CRAB was identified in this study and found to variably carry ompA and *csuE* genes, suggesting the importance of implementation of biofilm eradication practices to reduce the burden of infection in critical settings.

Keywords: Biofilm, Carbapenem-resistant *Acinetobacter baumannii*, CsuE, Microtiter plate assay, Outer membrane protein A

INTRODUCTION

Acinetobacter baumannii, an aerobic, non-fermenting, non-motile, and gram-negative-coccobacilli has emerged as an alarming pathogen globally. It causes infections such as bacteremia, secondary meningitis, ventilator-associated pneumonia, skin and wound infections, and catheter-associated urinary tract infections acquired nosocomially.[1]

This coccobacillus is receiving noteworthy attention by virtue of being an extensively drug-resistant pathogen to frequently used antimicrobial agents, in addition to carbapenems, limiting

the therapeutic options.[1] The upsurge of carbapenem-resistant *A. baumannii* (CRAB), categorized as a critical priority pathogen by the World Health Organization, forming biofilms in hospital environments is observed globally.[2] Assemblages of microorganisms enclosed in a matrix and forming biofilms function as an association providing protection for microbes, thus enhancing antibiotic resistance. This biofilm-forming ability aids in the increased transmissibility of multidrug-resistant organisms (MDRO) such as CRAB in healthcare settings and adds to the burden of infections.[3] The formation of biofilm is multifactorial and involves various elements such as collagenous adherence, material assembly, expression, and iron uptake.[4] Pili, which facilitates adhesion and development of biofilm, is also essential to produce biofilm formation in *A. baumannii*. Genes coding for biofilm formation are assembled together and arranged in a csu operon, the products leading to the formation of pilus-like bundles.[5] It has been observed that the development of strong biofilms on the plastic surface by *A. baumannii* 19606 is somewhat facilitated by outer membrane protein A, that is, abbreviated as ompA. This pathogen carries an adhesion molecule, that is, ompA, encoded by *ompA* gene. OmpA is essential during the attachment of the pathogen to human epithelial cells and further leads to biofilm formation.[3,5]

Biofilm formation significantly contributes to antibiotic resistance through mechanisms such as efflux pumps, enzyme modification, and reduced cell permeability. The primary concern with CRAB biofilms is their high tolerance to antimicrobial agents, which can lead to the development of antimicrobial resistance (AMR).[2] These resistance mechanisms within biofilms hinder effective bacterial eradication. In addition, biofilm formation helps bacteria survive in the host by increasing pathogen numbers and shielding them from the immune system.[4] Although biofilms are known to play a crucial role in the severity of hospital-acquired infections,[2,5] the relationship between biofilm formation and AMR in CRAB has not been extensively studied. To the best of our knowledge, no adequate studies have been conducted in our setting regarding AMR and biofilm formation. The rationale of the study was to unravel the relation of biofilm production with AMR. Therefore, the detection of biofilm formation is crucial to suggest appropriate therapeutic management of infections caused by these CRABs and further strengthen antimicrobial stewardship guidelines to limit further escalation of carbapenem-resistant variants among *A. baumannii* isolates.

Keeping the above in mind, this study was aimed at detecting the biofilm-producing CRAB using phenotypic microtiter plate assay and genotypic characterization of biofilm-forming genes and identifying possible relation, if any, between biofilm formation and AMR at our clinical setup.

MATERIALS AND METHODS

Study design

This observational study was at the microbiology department of a tertiary care center for a period of 1 year from March 2022 to March 2023 after obtaining the Institution's Ethical Clearance.

All consecutive isolates of CRAB recovered from various clinical specimens such as endotracheal (ET) secretion, tracheal secretion, pus, sputum, swab, bronchoalveolar lavage (BAL) fluid, and blood and central venous catheter (CVC) tip were included in the study. All other pathogens other than CRAB were excluded from the study. A total of 72 non-duplicate clinical isolates of CRAB were identified, and their antimicrobial susceptibility was determined using the VITEK-2 system (Biomerieux, UK). Wherever required, the Kirby–Bauer disc diffusion method was used for selected antimicrobials.[6]

Phenotypic assay of biofilm production

Microtiter plate assay

Producers of biofilm CRAB were detected using the microtiter plate method.[7] Overnight cultures of test organisms in trypticase soy broth were used to prepare standard inoculum, that is, 0.5 McFarland and diluted in 1:20. The wells of a 96-welled microtiter plate were dispensed with 200 mL of each bacterial suspension followed by an incubation of 24 h at 37°C. The suspensions were aspirated after incubation. Planktonic cells in wells were removed by 200 mL phosphate-buffered saline (PBS), followed by decanting and subsequently drying in the air for 15 min. 200 mL of crystal violet solution of 0.1% v/v used to stain the wells and incubated for 15 min at room temperature. Further, wells were washed with PBS to remove excess stains. Finally, 200 mL of acetic acid solution of 33% v/v was used to solubilize these stained wells and incubated at 37°C for 15 min. A microtiter plate reader (Bio-rad USA) was used to measure optical density at 630 nm. *A. baumannii* American Type Culture Collection19606 (ATCC19606) and one uninoculated well served as positive and negative controls, respectively. CRAB isolates were identified as biofilm producers based on optical density (OD) value. Mean absorbent values derived from triplicate wells were recorded as results.

Detection of biofilm-forming gene by polymerase chain reaction (PCR)

Deoxyribonucleic acid (DNA) was extracted from an overnight culture of each isolate using a bacterial genomic DNA extraction kit (Hi-media, India) under the

manufacturer's protocol. The ompA and csuE were detected using a primer[8] set, as mentioned in the supplementary material. A 20 µL reaction volume of PCR consisting of genomic DNA of 2 µL, Taq DNA polymerase (0.4 µL), deoxynucleoside triphosphate (dNTP) mix (1 µL), 2 µL of Taq buffer (consisting of $MgCl_2$), and 1 µL (10 pmol) of each primer was used. Conditions for the PCR of ompA were 94°C for 2 min, that is, initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, an annealing temperature of 52.1°C for 1 min, an extension at 72°C for 2 min, followed by a final extension at 72°C for 1 min. PCR for csuE was performed at 94°C for 2 min, that is, initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, an annealing temperature of 70.5°C at 1 min, an extension at 72°C for 2 min, followed by a final extension at 72°C for 1 min. Positive and negative controls were included in all PCR assays.

Statistical analysis

Data analyses were performed using IBM Statistical Package for the Social Sciences version 20 software. The Chi-square test was used to determine the relationship between AMR and biofilm formation.

RESULTS

Sample-wise distribution of CRAB isolates

Out of 72 CRAB isolates, CRAB was recovered most commonly from clinical specimens such as ET secretions (54%), followed by blood (18%) and tracheal tube secretions (10%), pus (6%), sputum (6%), swab (3%), CVC tip (3%), and BAL fluid (1%).

Table 1 depicts the antimicrobial susceptibility pattern of CRAB; out of 72 clinical test isolates of CRAB, a high level of resistance was observed against cefepime (100%), levofloxacin (99%), and ampicillin-sulbactam (96%). Table 2 shows the distribution of biofilm-forming genes among biofilm-producing and non-biofilm-producing CRAB clinical isolates; out of a total of 72 CRAB isolates, 64 (89%) were identified as biofilm producers. Out of 64 biofilm-producing CRABs, the majority were identified as strong biofilm formers (66%). Table 3 describes the relationship between biofilm producers and drug resistance. A high frequency of biofilm-forming CRAB was found resistant to levofloxacin (87.5%), ampicillin-sulbactam (86.1%), amikacin (86.1%), cotrimoxazole (62.5%), and minocycline (43.1%) in our study. Whereas non-biofilm-forming CRAB showed resistance against levofloxacin (11.1%), ampicillin-sulbactam (9.7%), amikacin (9.7%), minocycline (9.7%), and to cotrimoxazole (8.3%). This relation was found to be statistically insignificant.

Table 1: Antimicrobial susceptibility pattern of CRAB (*n*=72).

Antibiotic	Resistant (%)	Intermediate (%)	Sensitive (%)
Ampicillin-sulbactam	69 (96)	-	3 (4)
Cefepime	72 (100)	-	-
Levofloxacin	71 (99)	-	1 (1)
Amikacin	69 (96)	1 (1)	2 (3)
Cotrimoxazole	51 (70)	-	21 (29)
Minocycline	38 (53)	12 (17)	22 (31)

CRAB: Carbapenem-Resistant *Acinetobacter baumannii*

Table 2: Distribution of biofilm-forming genes among biofilm-producing and non-biofilm-producing CRAB clinical isolates.

Type of CRAB isolates	Percentage of isolates	ompA	csuE
Biofilm producers (*n*=64)			
Strong biofilm producers	42 (66)	38	26
Moderate biofilm producers	20 (31)	16	17
Weak biofilm producers	2 (3)	2	2
Non-biofilm producers (*n*=72)	8 (11)	8	4

CRAB: Carbapenem-Resistant *Acinetobacter baumannii*, ompA: Outer membrane protein A, csuE: chaperon-usher pilus E.

DISCUSSION

The global rise of multidrug-resistant CRAB isolates is a major concern in clinical settings. Most commonly, CRAB was recovered from the lower respiratory tract such as ET secretions (54%) in our study. In a similar study by Al-Rashed *et al.*, 32% of multidrug resistance (MDR) *A. baumannii* was isolated from ET secretions.[9] All of the CRAB isolates (100%) were identified as MDROs as it was found that they were resistant to three or more antibiotics. These findings are consistent with other similar studies from Sudan and India, where multidrug-resistant CRAB prevalence was reported as high as 97% and 91%, respectively.[10,11] It was found that these CRAB clinical isolates showed resistance against both imipenem and meropenem (100%). In the study by Agarwal *et al.*, a high resistance rate of 90.5% and 95.2% was observed against imipenem and meropenem, respectively.[12] The reason behind high resistance in *Acinetobacter* to carbapenem drugs such as meropenem is suggested to acquiring resistant determinants from the atmosphere in response to selective pressure and intrinsic ability to quickly utilize the efflux pumping mechanism.[13,14]

As a non-polymyxin-based agent, minocycline has shown potential effect in the treatment of CRAB infections, and

Table 3: Comparisons of drug resistance among biofilm-forming and non-biofilm-forming CRAB isolates.

Antibiotics	No. of drug-resistant non-biofilm-forming CRAB (*n*=8) (%)	No. of drug-resistant biofilm-forming CRAB (*n*=64) (%)	*P*-value
Ampicillin sulbactam	7 (87.5)	62 (96.9)	0.754*
Amikacin	7 (87.5)	62 (96.9)	0.301
Levofloxacin	8 (100)	63 (98.4)	0.889*
Cotrimoxazole	6 (75)	45 (70.3)	1.000*
Minocycline	7 (87.5)	31 (48.4)	0.163**

An asterisk (*) indicates Yates test and **indicates Fisher's Exact test. CRAB: Carbapenem-resistant *Acinetobacter baumannii*

minocycline, tigecycline, cefiderocol, or colistin combined with carbapenems may be used as CRAB treatments. The results of our study demonstrated significant *in vitro* activity of cotrimoxazole and minocycline against CRAB isolates, supporting their usage and inefficient management of these isolates. Out of 72 CRAB isolates, 29% were found sensitive to cotrimoxazole and 31% sensitive to minocycline. In various clinical studies/systematic reviews, it has been suggested that minocycline showed 73% successful clinical outcomes. Raz-Pasteur *et al*. reported that cotrimoxazole showed appropriate activity against CRAB-infected patients. In conclusion, cotrimoxazole and minocycline might be a valuable treatment option against CRAB.[15-17]

Numerous studies have found that *A. baumannii* is prevalent in adverse environments and resistant to several antimicrobial agents by their ability to form biofilm.[3,5] According to our results, among biofilm-producing CRAB clinical isolates (89%), 66% of CRAB showed a strong ability to form biofilms. Pattnaik and Banashankari have reported 64% of *A. baumannii* isolates as biofilm producers in their findings which are consistent with our results.[7] In studies using similar methods of biofilm detection by microtiter plate method, 100% and 70.1% of *A. baumannii* isolates were identified as biofilm producers.[18,19] The presence of foreign devices such as ET tubes and catheters, residence in an intensive care unit prolonged hospitalization, prolonged mechanical ventilation, and high colonization pressure contribute to biofilm formation. Our study reports, that out of 64 biofilm formers CRAB, isolates recovered from ET secretions (n = 36, 52%) were the predominant biofilm formers. It is similar to the previous study by Guddeti *et al*.[20]

A. baumannii ompA (AbOmpA) is essential for eukaryotic cell adherence, acts as a porin, and also contributes to biofilm formation and serum resistance.[21] In the present study, we detected the presence of ompA (89%) and csuE (68%) in CRAB test isolates. Yang *et al*. also observed a high prevalence of biofilm-forming genes in AbOmpA (91.6%) and csuE (68.8%).[22] In a similar study, Khoshnood *et al*. found both ompA and *csuE* genes (86%) among MDR *A. baumannii*.[23] In a study by Liu *et al*., the predominant gene was ompA (100%) among *A. Baumannii*.[8] In our study, all non-biofilm-producing CRAB isolates (11%) were also carrying biofilm-forming genes in variable distributions; these findings are concordant with the Yang *et al*. study.[22]

Biofilms on surfaces lead to decreased penetration of antibiotics, which makes managing infections challenging. In a similar study, a positive relationship between biofilm formation and antibiotic resistance among *A. baumannii* has been reported.[22] Our study found that strong biofilm formers are resistant to antibiotics such as β-lactam inhibitors, that is, ampicillin-sulbactam (95%), fourth-generation cephalosporin (cefepime) 100%, amikacin (98%), and levofloxacin (100%). Previous evidence reported in the literature that associations between biofilm formation and reduced antimicrobial function cannot be explained by conventional mechanisms of development of resistance.[22] Numerous mechanisms, such as biofilm phenotype adaptive mechanisms, enzyme-caused neutralizations, limited diffusion, and slow growth rates, are considered key factors in the high resistance among biofilm formers. Our study shows both biofilm formers and non-biofilm forming to be multidrug-resistant, the relationship between drug resistance and biofilm-forming was, however, found to be statistically insignificant, which may be due to the small sample size. In a similar study, Amin *et al*. also reported an insignificant relationship between the ability to form biofilm and resistance among clinical isolates of *A. baumannii*.[24] Baidya *et al*. also showed a high prevalence of MDR in biofilm-producing gram-negative bacteria, but no significant relationship between biofilm formation and multidrug resistance was observed.[25] However, Chukamnerd *et al*. found a borderline significant association between biofilm formation and AMR.[26]

Biofilm formation and multidrug resistance may arise from independent mechanisms. Biofilms create a physical barrier that can hinder antibiotic penetration. Multidrug resistance typically arises from genetic mechanisms, such as the presence of efflux pumps and chromosomal mutations. These two phenomena might not always correlate directly. MDR in CRAB is predominantly driven by biofilm-independent mechanisms, including horizontal

gene transfer (e.g., plasmids carrying resistance genes) or intrinsic resistance traits that do not involve biofilm formation.[27]

Some limitations in our study should be described. First, the study population was relatively small, so the lack of a significant association between biofilm formation and AMR in CRAB could be attributed to the small sample size. Second, the study was conducted at a single tertiary care hospital, so the results may not apply to other regions. Finally, our study did not explore potential genetic factors that could explain the phenotypic findings related to biofilm formation and AMR. Our study highlights the importance of the detection of biofilm production among CRAB at clinical setup.

CONCLUSIONS

A high frequency of biofilm-forming CRAB was identified in this study, and higher expression of biofilm-forming genes such as ompA and csuE was found among these biofilm-producing MDROs. The relationship between biofilm formation and drug resistance was found to be statistically insignificant. Further investigations on how to manage biofilm-producing CRAB are needed. The findings of this study emphasize the urgent need for the detection of other drug-resistance mechanisms to guide effective therapeutic management of infection caused by these biofilms-producing and non-biofilm-producing drug-resistant MDROs. This study advocates further research into convenient methods for detecting biofilm both *in vivo* and *in vitro* in routine laboratory settings, as well as exploring alternative treatments for biofilm-associated pathogens to reduce the risk posed by resistant bacteria.

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