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Original Research Article

Molecular detection of virulence genes from carbapenem-resistant biofilm-forming *Acinetobacter baumannii* isolated from a tertiary care multispecialty hospital in Pune, India

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Abstract

Background: *Acinetobacter baumannii (A. baumannii)* has globally emerged as an important pathogen in hospital-acquired infections. Bacterial pathogenesis is associated with numerous virulence factors, such as colonization, invasion, biofilm formation, antimicrobial resistance, etc. The study investigated the antibiotic susceptibility pattern, biofilm formation capabilities, and presence of virulence genes in clinical isolates of *A. baumannii*.

Materials and Methods: Out of 115 collected isolates, 32 carbapenem-resistant biofilm-forming extensively drug-resistant *A. baumannii* (XDR-AB) were included in this study. The isolates were identified by VITEK 2 and confirmed by polymerase chain reaction (PCR) targeting the ^{bla}OXA-51 gene. Antibiotic susceptibility pattern was determined by VITEK 2. All the isolates were examined for biofilm formation using the tube method, and PCR was performed to detect virulence genes.

Results: All study isolates were carbapenem-resistant XDR-AB and showed a high resistance rate (84.38%-100%) to most of the antibiotics except colistin and minocycline. Biofilm formation was documented in 100% (32/32) of isolates and categorized as strong, moderate, and weak biofilm formation in 7 (21.88%), 12 (37.5%), and 13 (40.62%) isolates, respectively. Out of 32 isolates, 30 (93.75%) harbored virulence genes, including Bap (30, 93.75%), OmpA (29, 90.62%), cnf1 (7, 21.87%), and csgA (5, 15.62%). The study also reported the presence of multiple genes in a single isolate (30/32, 93.75%).

Conclusion: The study highlights that colistin and minocycline are effective antibiotics for treating carbapenem-resistant biofilm-forming XDR-AB infections, which can be included in our hospital antibiotic policy. However, the combination therapy of colistin with minocycline can have better results compared to monotherapy. Nearly one-fourth (21.88%) of the biofilm-forming isolates were strong biofilm producers. The study also documented a high prevalence (93.75%) of virulence genes and the existence of genetic heterogeneity in study isolates that can trigger further dissemination of the gene. Therefore, molecular surveillance of local isolates for the virulence factor genes is crucial for containing transmission, diseases, and outbreaks.

Keywords: *Acinetobacter baumannii*, Hospital-acquired infections, Antibiotic resistance, Biofilm, Virulence genes.

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1. Introduction

Acinetobacter baumannii (A. baumannii), a gram-negative coccobacillus, poses a serious threat to healthcare facilities worldwide. It is one of the major causative agents in hospital-acquired infections (HAIs), particularly predominant in intensive-care units (ICUs). It causes various infections such as pneumonia, bacteraemia, meningitis, endocarditis, wound infection, etc.[1] Approximately 1 million people are infected with *A. baumannii* each year globally.[2] The high burden of the disease is mainly due to the increased rate of resistance shown by the organism. *A. baumannii* is frequently resistant to commonly used antibiotics.[3] Earlier, carbapenems were the drug of choice for the treatment of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *A. baumannii* (XDR-AB). However, nowadays, it is no longer effective due to the emergence of carbapenem-resistant *A. baumannii* (CRAB). The World Health Organization (WHO) categorized CRAB as a priority 1 (“critical group”) pathogen that urgently requires novel antimicrobial therapeutic strategies.[4] The pathogenicity of this notorious pathogen is attentively associated with various virulence factors.[5] Among these, the formation of biofilm is a crucial contributor.[6] Other

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virulence factors, including adhesion, invasion, outer membrane proteins, cytotoxic necrotizing factors, curli fibers, etc., also contribute to bacterial pathogenicity.[5] Clinical isolates of *A. baumannii* harbored various virulence factor genes such as Bap (biofilm-associated protein), OmpA (outer membrane protein A), cnf1 (cytotoxic necrotizing factor 1), csgA (curli-specific gene A), etc. Virulence genes contribute to bacterial colonization, infections, persistence in the host cell, and antimicrobial resistance.[5,7] Therefore, assessing the virulence genes present in *A. baumannii* is important to monitor the disease transmission and formulate strategies for prevention. Hence, the study aimed to evaluate antibiotic susceptibility patterns, biofilm formation, and virulence gene detection in clinical isolates of *A. baumannii*.

2. Materials and Methods

2.1. Study settings and isolate selection

The cross-sectional observational single-centre study was conducted in the Department of Microbiology at a tertiary care teaching multispecialty hospital in Pune, India, from August 2022 to May 2023 after getting approval from the institutional ethics committee. A total of 115 clinical isolates of *A. baumannii* were collected from various specimens like endotracheal secretion (ET), blood, pus, body fluids etc. Of the total isolates, 32 carbapenem-resistant biofilm-forming XDR-AB were included in this study, although repeated isolates from a single patient were excluded.

2.2. Isolation and identification of A. baumannii

Based on the type of specimens, culture media such as Blood agar, McConkey agar, and Cystine-lactose-electrolyte-deficient agar (HiMedia Laboratories, Mumbai, India), were selected for the isolation and identification of the organism. Specimens were cultured on media and incubated for 18–24 hours at 37°C. Following incubation, the initial identification was done by conventional microbiological techniques like colony morphology examination, gram staining, and biochemical tests.[8] Further, the isolates were verified by VITEK 2 (bioMérieux, France), and the final identification was made by detecting the ^{bla}OXA-51 gene (**Figure 1**) intrinsic to *A. baumannii*.[9] The PCR condition for the ^{bla}OXA-51 gene detection is mentioned in **Table 1**.

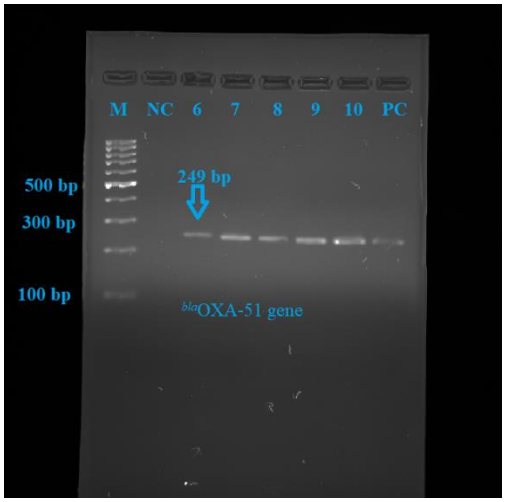

Figure 1: Gel image of PCR products of ^{bla}OXA-51 gene (249 bp). M (marker 100bp), NC (Negative control), Lanes 6-10 (Test isolates), PC (Positive control)

2.3. Antibiotic susceptibility testing

The antibiotic susceptibility was tested by VITEK 2,[10] and the report interpretation was done according to the Clinical and Laboratory Standards Institute (CLSI) 2021 guidelines. In this study, antibiotics used for susceptibility testing are listed in **Table 2**. To determine carbapenem resistance, imipenem and meropenem minimum inhibitory concentrations (MIC) of ≥8µg/ml were taken into consideration. The broth microdilution test further confirmed colistin susceptibility. *A. baumannii*, non-susceptible to at least one agent in three or more (≥3) antimicrobial categories, was considered MDR while non-susceptible to at least one agent in all but two or fewer antimicrobial categories, which suggests that bacterial isolates remain susceptible to only one or two categories was considered XDR.[11]

Table 1: PCR conditions to amplify the gene

Gene	Stage 1	Stage 2				Stage 3
	Initial denaturation (Temperature and Time)	Cycles	Denaturation	Annealing	Extension	Final extension (Temperature and Time)
			(Temperature and Time)			
^{bla}OXA-51	95°C for 5 minutes	35	95°C for 20 seconds	55°C for 45 seconds	72°C for 45 seconds	72°C for 3 minutes
Bap, OmpA	95°C for 1 minute	40	95°C for 15 seconds	60°C for 15 seconds	72°C for 3 seconds	72°C for 3 minutes
cnf1, csgA	95°C for 4 minutes	30	95°C for 50 seconds	58°C for 60 seconds	72°C for 45 seconds	72°C for 8 minutes

Table 2: Antibiotic susceptibility pattern of isolates (n=32)

Name of Antibiotics	Sensitive, n (%)	Intermediate, n (%)	Resistant, n (%)
Piperacillin/Tazobactam	0	0	32 (100)
Ceftazidime	0	0	32 (100)
Cefoperazone/Sulbactam	2 (6.25)	3 (9.37)	27 (84.38)
Cefepime	0	1 (3.12)	31 (96.88)
Imipenem	0	0	32 (100)
Meropenem	0	0	32 (100)
Amikacin	0	0	32 (100)
Gentamicin	0	0	32 (100)
Ciprofloxacin	0	0	32 (100)
Levofloxacin	0	2 (6.25)	30 (93.75)
Minocycline	8 (25)	5 (15.62)	19 (59.38)
Colistin	32 (100)	0	0
Trimethoprim/Sulphamethoxazole	2 (6.25)	0	30 (93.75)

2.4. Detection of biofilm formation:

The tube method was used to detect biofilm formation.[12] Clinical isolates were inoculated into a tube containing 5 ml of trypticase soy broth and incubated for 24 hours at 37°C. Following incubation, the growth of the organisms appeared as turbid form. After that, the tubes were decanted, cleaned with phosphate buffer saline (pH 7.2), and left to air dry. After being dried, each tube was stained with 0.1% solution of crystal violet and washed with deionized water. Then, it was kept in an inverted position to dry again, and biofilm formation was observed (**Figure 2**). The test was performed in triplicate. Strains of *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as positive and negative controls, respectively. Depending on the results of quality control strains, interpretation was done for biofilm formation. When a visible layer formed on the tube's walls and base, the isolate was considered positive for biofilm formation; however, the absence of such layer suggested no biofilm formation. Four possible scores determined the amount of biofilm formed: (4) strong positive, (3) moderate positive, (2) weak positive, (1) negative.

Figure 2: Biofilm formation of test isolates. Tube 1: PC (Positive control), Tube 2: NC (Negative control), Tube 3: Strong positive, Tube 4: Moderate positive, Tube 5: Weak positive, Tube 6: Negative biofilm formation

2.5. Molecular detection of virulence genes

The DNA of *A. baumannii* was extracted by using NucleoSpin (MACHEREY-NAGEL, Germany) extraction kit. PCR was performed to detect virulence genes (Bap, OmpA, cnf1, and csgA) from template bacterial DNA prepared by the boiling method.[13] Briefly, in the PCR technique, 5 µl of template DNA was mixed in the 20 µl of reaction volume, and specific primers were used for targeted genes. Cyclic conditions in the PCR process are mentioned in **Table 1**. In PCR, previously confirmed CRAB and *Escherichia coli* ATCC 25922 strains were used as positive and negative controls, respectively. Following PCR cycles, amplicons were examined by gel electrophoresis. Agarose gel (2%) containing 5 µg/ml ethidium bromide (HiMedia) was prepared and run at 100 v for 45 minutes. A gel documentation system (Bio-Rad Laboratories, USA) was used to visualize images of Bap and OmpA (**Figure 3**) as well as cnf1 and csgA genes (**Figure 4**).[14] A 100 bp DNA ladder (Thermo Fisher Scientific, USA) was used as a molecular marker to assess the size of PCR amplicons.

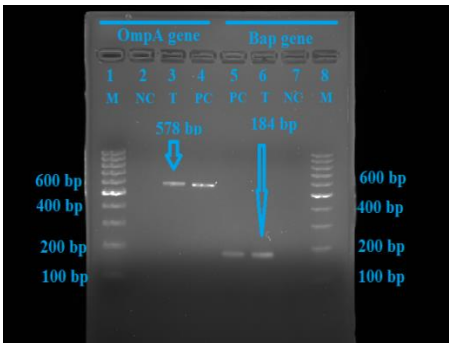

Figure 3: PCR products of OmpA gene (578 bp) and Bap gene (184 bp). Lanes 1-4 represent OmpA gene (Lane 1: M-Molecular marker 100bp, Lane 2: NC-Negative control, Lane 3: T-Test isolate, Lane 4: PC-Positive control). Lanes 5-8 represent Bap gene (Lane 5: PC-Positive control, Lane 6: T-Test isolate, Lane 7: NC-Negative control, Lane 8: M-Molecular marker 100bp)

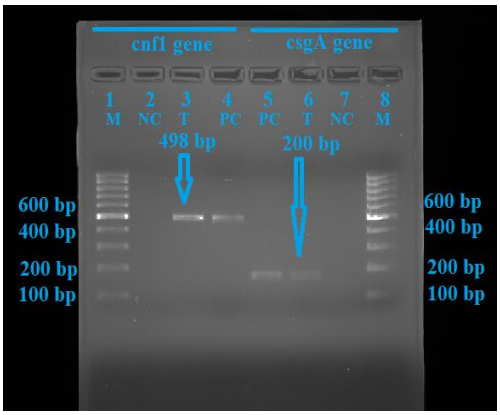

Figure 4: PCR products of cnf1 gene (498 bp) and csgA gene (200 bp). Lanes 1-4 represent cnf1 gene (Lane 1: M-Molecular marker 100bp, Lane 2: NC-Negative control, Lane 3: T-Test isolate, Lane 4: PC-Positive control). Lanes 5-8 represent csgA gene (Lane 5: PC-Positive control, Lane 6: T-Test isolate, Lane 7: NC-Negative control, Lane 8: M-Molecular marker 100bp)

2.6. Statistical analysis

Data were analyzed using statistical software SPSS version 27.0 (IBM Corp., Chicago, IL, USA). Continuous variables were described as mean±SD. Frequencies and percentages were used to present categorical variables.

3. Results

3.1. Demographic and epidemiological details of study participants

This study investigated 32 isolates of carbapenem-resistant biofilm-forming XDR-AB. Isolates from respiratory specimens were 16 (50%), followed by blood 11 (34.38%), body fluids 4 (12.5%), and pus 1 (3.12%). The mean age of the study participants was 53.9±17.6 years, and 14 (43.75%) participants were observed at 60 years old or above. Male and female participants were 12 (37.5%) and 20 (62.5%). The average duration of hospitalization was 21 days, and 18 (56.25%) participants were documented in co-morbid conditions.

3.2. Antibiotic susceptibility testing

The susceptibility to antibiotics of 32 isolates is shown in **Table 2**. All the isolates were XDR, 100% resistant to carbapenems (imipenem, meropenem). However, the minocycline resistance rate was documented at 59.38% (19/32), and none of the isolates (0/32) were resistant to colistin.

3.3. Detection of biofilm formation

Biofilm formation was noticed in all the study isolates (n=32). Among these, strong (score 4), moderate (score 3), and weak (score 2) biofilm producers were 7 (21.88%), 12 (37.5%), and 13 (40.62%), respectively.

3.4. Molecular detection of virulence genes

The confirmatory ^{bla}OXA-51 gene was present in all the isolates (32/32, 100%), and virulence genes were detected in 30 (93.75%) isolates. Out of the total 32 isolates, virulence genes such as Bap, OmpA, cnf1, and csgA were detected in 30 (93.75%), 29 (90.62%), 7 (21.87%), and 5 (15.62%) isolates, respectively. The detection of multiple genes in a single isolate of *A. baumannii* was documented in 30 isolates (**Table 3**).

Table 3: *Acinetobacter baumannii* harboring multiple virulence genes in a single isolate (n=30)

Genes	Number of isolates (%)
Bap and OmpA	23 (76.67)
Bap and cnf1	1 (3.33)
Bap, OmpA and cnf1	1 (3.33)
Bap, OmpA, cnf1 and csgA	5 (16.67)

4. Discussion

The study described the demographic and epidemiological features of the participants. All the isolates (n=32) were obtained from ICUs, and the majority (16/32, 50%) were from respiratory specimens, similar to previous studies.[2,9] Endotracheal secretion was the main source (13/32, 40.62%) of the specimens. A significant number (14/32, 43.75%) of the study participants were 60 years old or above, and females (20/32, 62.5%) were more compared to male participants (12/32, 37.5%). In contrast, Dey S. et al. reported male predominance (76%) in their study.[15] The study also documented that more than half of the participants (18/32, 56.25%) were in co-morbid conditions, like diabetes mellitus, hypertension, chronic kidney disease, etc.

Antimicrobial resistance (AMR), a “silent pandemic,” has now emerged as one of the greatest concerns globally and is estimated to cause approximately 10 million deaths per year by 2050 if sufficient preventive measures are not taken.[16] Many bacterial species have developed extensive resistance to almost all classes of antibiotics. *A. baumannii* also epitomizes the trend and shows high resistance to commonly used antibiotics. The present study report of antibiotic susceptibility test revealed that *A. baumannii* was 100% resistant to ceftazidime, piperacillin/tazobactam, imipenem, meropenem, amikacin, gentamicin, and ciprofloxacin. The study also documented a high resistance rate to cefepime (96.88%), levofloxacin (93.75%), and trimethoprim/sulphamethoxazole (93.75%); a moderate resistance rate to cefoperazone/sulbactam (84.38%), and a low resistance rate to minocycline (59.38%). All the isolates (32/32, 100%) were sensitive to colistin. Likewise, other studies have previously documented high levels of antibiotic resistance against *A. baumannii*.[1,9] Currently, the therapeutic option for drug-resistant *A. baumannii* is limited. Based on the antibiotic susceptibility pattern of the pathogen, the study

suggests that colistin and minocycline may be effective treatment options for CRAB infections. Although colistin is widely accepted as a treatment option, its uses raise concerns due to the high risk of nephrotoxicity. In contrast, minocycline has a suitable pharmacokinetic and pharmacodynamic profile. However, the combination therapy of colistin and minocycline can achieve a better treatment outcome compared to monotherapy.[17]

Biofilm formation plays a vital role in the pathogenesis of *A. baumannii*, particularly in device-related infections and the development of drug resistance. The biofilm formation by *A. baumannii* helps to withstand extreme circumstances in the hospital environment and resist antibiotic effectiveness.[6,12,18] The present study documented that the rate of strong, moderate, and weak formation by *A. baumannii* was 21.88%, 37.5%, and 40.62%, respectively. In contrast, a study conducted by AM Alamri et al. reported a lower rate of (20.8% and 29.5%) strong and moderate biofilm formation and a higher rate (49.7%) of weak biofilm formation in *A. baumannii* compared to the present study.[19]

The various virulence factor genes present in *A. baumannii* influence disease progression to complications. Isolates of *A. baumannii* have the Bap gene, which encodes a very large surface protein called biofilm-associated protein. Bap secretion helps the pathogen adhere to the host cell and contributes to biofilm formation, maturation, and maintenance. Therefore, it has a significant role in the persistence of the pathogen in the hospital environment, infections, and antibiotic resistance.[9,20] The OmpA is another factor contributing to the virulence mechanism of bacteria. It is one of the most important outer membrane proteins in *A. baumannii*, which plays a versatile role in bacterial pathogenesis, including attachment to the host cell surface, invasion, autophagy induction, cellular injury, etc.[5] Additionally, it also takes part in bacterial survival, biofilm formation, and antibiotic resistance.[7] The present study reported a high prevalence of Bap and OmpA genes in *A. baumannii* isolates. In particular, Bap and OmpA genes were detected in 93.75% and 90.62% of isolates, respectively. Our findings are consistent with previous studies that reported a high frequency of Bap and OmpA genes.[14,21-23] The increased frequency of Bap and OmpA genes in this study indicates that both genes play a vital role in biofilm formation and antibiotic resistance. *A. baumannii* strains are also known to harbor cnf1 and csgA virulence factor genes, which are mainly responsible for bacterial adhesion, colonization, and invasion into the target organs. Cnf1 encodes cytotoxic necrotizing factor, which has the potential to stick at the site of infections, reduce the number of phagocytic cells, and prevent wound healing.[13] In the process of biofilm formation by *A. baumannii*, an extracellular matrix consisting of curli amyloid fibers and cellulose is produced. CsgA (curli-specific gene A), a major subunit of the curli amyloid fiber, enhances curli fiber production and contributes to bacterial adhesion to surfaces, invasion, cell aggregation, and biofilm formation.[24] The study reported that the prevalence rate of the cnf1 and csgA genes in *A. baumannii* was 21.87% and 15.62%, respectively. In contrast, a study conducted by AL-Kadmy et al. documented a higher frequency of cnf1 (47.6%) and csgA (66.7%) genes in *A. baumannii* isolates.[13]

Overall, our study noted a very high prevalence (30/32, 93.75%) of virulence genes in the clinical isolates of *A. baumannii*. No gene was detected in 2 (6.25%) isolates. Bap was the most commonly identified (93.75% of isolates) virulence gene, and more than one gene in a single isolate was documented in all virulence gene-positive isolates (30/32, 93.75%). The coexistence of virulence genes in a single isolate was found with various combinations, such as Bap with OmpA (23/30, 76.67%), Bap with cnf1 (1/30, 3.33%), Bap with OmpA, and cnf1 (1/30, 3.33%) and Bap with OmpA, cnf1 and csgA (5/30, 16.67%) suggesting the existence of genetic heterogeneity in the clinical isolates of *A. baumannii*. Genetic heterogeneity in the isolates may further transmit the gene to other isolates of the same species or different bacteria.

The study has a few limitations that need to be mentioned. First, it was a single-centre study with relatively small specimen sizes. Second, other genes associated with bacterial virulence and antibiotic resistance were not included. Therefore, multi-centre molecular studies with large specimen sizes are suggested for further research.

5. Conclusion

Colistin and minocycline are effective drugs for the treatment of carbapenem-resistant biofilm-forming XDR-AB infections. This study's findings can make an important contribution to our hospital's antibiotic policy. However, instead of monotherapy, combination therapy of colistin with minocycline may have a better outcome. Among the isolates that formed biofilms, nearly one-fourth (21.88%) were strong biofilm producers, suggesting their role in prolonged hospitalization. The study identified a high prevalence of virulence genes along with genetic heterogeneity (presence of more than one gene) in the isolates of *A. baumannii*. Therefore, detection of virulence factor genes is important in addition to effective infection control measures for the prevention of *A. baumannii* infections. Furthermore, comprehensive molecular surveillance of various virulence genes implicated in the pathogenic mechanisms is suggested to avert the further dissemination of the gene in hospital environments.

6. Ethical Approval

This study was approved by institutional ethical committee with reference number DYPV/EC/775/2021.

7. Source of Funding

None.

8. Conflict of Interest

The author declares no conflict of interest.

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