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RESEARCH ARTICLE

A CROSS SECTIONAL STUDY TO CORRELATE BIOFILM FORMATION ALONG WITH BAP GENE AND FLUOROQUINOLONE RESISTANT GENE IN MDR ACINETOBACTER BAUMANNII IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Aim:To investigate the correlation between biofilm formation and its potential association with genes involved in biofilm production and antibiotic resistance mechanisms in MDR *A. baumannii*clinical isolates. Through phenotypic assays and molecular analysis, we characterized biofilm production and determined antibiotic susceptibility profiles. **Methods:** A total of 50 isolates of *Acinetobacter baumannii* from endotracheal aspirates of the ICU patients' samples received at the Department of Microbiology, BMCRI were collected and identified by VITEK 2 automated system as per laboratory protocol.Antimicrobial Susceptibility Testing was performed as per CLSI 2023 guidelines using Kirby bauer disc diffusion & vitek2 system. The biofilm production capability of the isolates was evaluated by qualitative tube method and bap gene &gyr A gene were detected by PCR. **Result:** *Acinetobacter baumannii* isolates were predominantly found in Endotracheal aspirates. A total of (41/50)82.5% isolates were MDR organisms.(39/50)78%isolates formed biofilms. (24/39) isolates (61.5%) were strong biofilm former, (9) weakly adherent, (6) non-biofilm producers.36 isolates (72%) showed the presence of bap gene of which 5 isolates were negative for biofilm producers and 30 isolates (60%) showed presence of gyr A gene of which 3 isolates were negative for biofilm producers and all were multi drug resistant. **Discussion and Conclusion:** The study concludes that there is a positive correlation between biofilm formation and associated genes in *A. baumannii*, the frequency of bap gene and gyrA gene were 72 % and 60% respectively. Statistical analysis revealed a significant correlation between frequency of gyr A gene and biofilm formation (p=0.017) and significant correlation between bap gene and biofilm formation (p= <0.001). Biofilm production promotes increased colonization and persistence leading to higher rates of device related infections. Strict adherence to infection control practices, such as hand hygiene, environmental cleaning and patient isolation can help mitigate the spread of these resistant organisms.

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INTRODUCTION

Acinetobacter baumannii, a gram negative non fermenter organism, has emerged as one of the most troublesome pathogens for health care institutions globallyThis organism commonly targets the most vulnerable hospitalized patients causing pneumonia particularly in patients on mechanical ventilation, it accounts for about 10% of all nosocomial infections and its propensity to cause large often multifacility nosocomial outbreaks [1]. Mortality due to *A. baumannii* infections can be as high as 75%and often difficult to treat due to high level of resistance to multiple Antibiotics as a result of both intrinsic and acquired mechanisms [2]. Biofilm formation, a complex process crucial for bacterial survival and persistence, is often mediated by various genes, including the Biofilm -associated protein (bap) gene.

Additionally, the emergence of fluoroquinolone resistance further complicates treatment strategies against *A. baumannii* infections. Understanding the correlation between biofilm formation and associated genes it is essential to develop effective therapeutic interventions against MDR *A. baumannii* infections. This study aims to explore the intricate relationship between biofilm formation, bap gene expression and fluoroquinolone resistance in MDR *A. baumannii* strains, shedding light on potential targets for combating these resilient pathogens.

MATERIALS AND METHODS

Sample Material: The type of specimen includes Endotracheal aspirate samples.

Sample Collection

- ET aspiration done with a sterile technique using 22 inch, 12F suction catheter
- Catheter introduced through the endotracheal tube for at least 30cm
- Gentle aspiration was performed without saline the first aspirate is discarded
- Second aspirate was collected after tracheal instillation of 5ml saline solution in sterile container & sent to lab within 1 hour of collection.

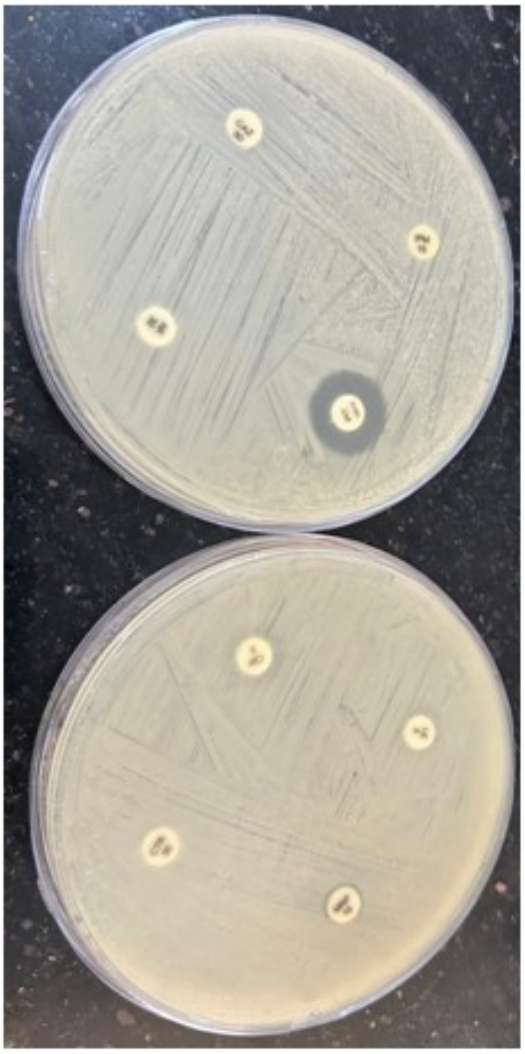
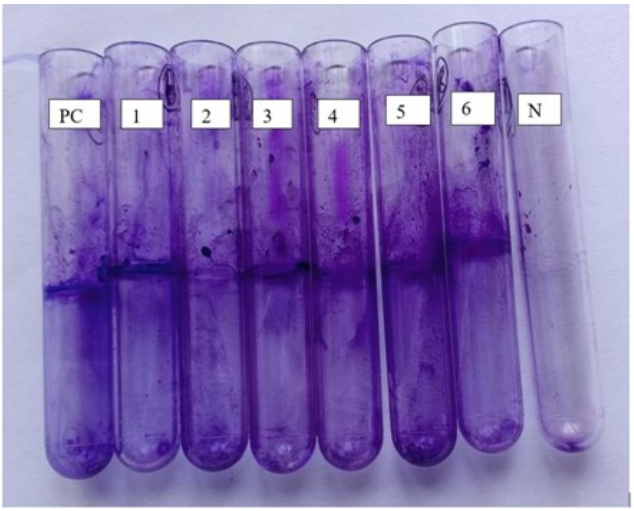


Figure 1: Mueller Hinton agar plate inoculated with a bacterial suspension for antimicrobial susceptibility testing for relevant antibiotics by Kirby Bauer disk diffusion method.

A cross sectional study conducted in department of Microbiology, BMCRI for 3 months duration from January 2024 to March 2024, Of the 70specimens collected from endotracheal aspirates of the patients hospitalized at ICU in the hospitals attached to BMCRI, who had been hospitalized for at least 48 hours under aseptic precautionsand sent to Infosys laboratory Victoria hospital for further processing, 50 strains of *A. baumannii* were selected for further studies.All the isolates were characterised to the species level by automated Vitek 2 systemand were tested for antimicrobial susceptibility testing for relevant antibiotics by Kirby Bauer disk diffusion method according to norms of Clinical Laboratory Standards Institute (CLSI) 2023 and Vitek 2 system. The antibiotic susceptibility was tested for following drugs listed amikacin(30µg), ceftazidime(30µg), ceftriaxone (30µg), ciprofloxacin (5µg), piperacillin-tazobactam (PTZ: 100/10 µg), imipenem(10µg), levofloxacin (5 µg), which were obtained from Hi-Media [2]. The isolates were cultured on Mueller Hinton agar plates inoculated with a bacterial suspension equal to 0.5 McFarland and incubated at 37°C for 24 h. Diameters of the zone of inhibition were interpreted with reference to the CLSI to determine whether the bacterium was susceptible (S), intermediate (I), or resistant (R) (Figure 1), to the tested drugs the isolates that were resistant to atleast three classes of antibiotics was considered as MDR *A. baumannii* as described [3]. *A. baumannii* ATCC 19606 was used as the positive control.

Biofilm formation: The biofilm formation potential of *A. baumannii* isolates was determined by qualitativetestusing tube method [2]. Fresh bacterial growth was sub-cultured in 5 mL of trypticase soy broth with loopful of organismand incubated for 24 h at 37 °C. Following incubation, the tubes were washed with phosphate-buffer saline (PBS; pH 7.2), dried in an inverted position and stained with 1% crystal violet for 10 min. The tubes were rinsed again in deionized water and air dried in inverted position, the evidence of biofilm formation is indicated by the appearance of visible film lining the wall and bottom of the tube. Based on the intensity of the color formed, biofilm formation was scored as negative/weak (-/+), moderately positive (++), and strongly positive (+++) (Figure 2)



PC- positive control *A.baumannii* ATCC 19606; NC- Negative control non biofilm producer *Escherichia coli;* Tube 1,3,4,5,6 – Strong biofilm producing *Acinetobacter* isolates; Tube 2 – Weak biofilm producer.

Figure 2. Biofilm formation on surfaces of tube at liquid- air interface

Detection of bap gene: DNA was extracted from overnight cultures of *A. baumannii*isolates using crude method of extraction were colonies from fresh culture emulsified in 300µl of distilled water vortex for about 5-10 sec, spin it for about 5sec incubate it in water bath at 100°C for 15 minutes followed by centrifugation at 11000rpm for 15 minutes supernatant was collected and used as DNA template pellet was discarded. Detection of bap gene by PCR technique. Thelist of primers used in the present study has been shown in (Table 1**).**

Table 1. Sequences of primers used in this study for detection of fluoroquinolone resistance and biofilm related genes

Target gene		Sequences (5'- 3')	Amplication size(bp)	Ref
bap	F	TGCTGACAGTG ACGTAGAACCACA	184	(4)
	R	TGCAACTAGTGG AATAGCAGCCCA		
gyr A	F	AAATCTGCCC GTGTCGTTGGT	343	(3)
	R	GCCATACCTAC GGCGATACC		

Table 2 & 3. shows the correlation with biofilm producing isolates, bap gene and gyr A gene

Biofilm	Bap gene Negative No of isolates	Positive No of isolates	Total No of isolates Biofilm producer
Negative	9	2	11
Positive	5	34	39
Total	14	36	50

Biofilm	Gyr A gene Negative No of isolates	Positive No of isolates	Total No of isolates Biofilm producer
Negative	8	3	11
Positive	12	27	39
Total	20	30	50

Table 4. Antibiotic susceptibility results (percentage) of the *A. baumannii* isolates

ANTIBIOTICS	Resistance in biofilm positive isolates (n=39)	Resistance of all isolates (n=50)
Amikacin	76.9	76.4
Ceftazidime	84.6	84.3
Ceftriaxone	82.0	82.3
Ciprofloxacin	84.6	84.3
Piperacillin tazobactam	82.0	82.3
Imipenem	76.9	76.4
Levofloxacin	76.9	78.4

The PCR were carried out in 25µL reaction volume consisted of 4.5µL of extracted DNA, 12.5 µL PCR Master mix, 0.5 µL(50pmol) of each primer, 7µl of Nuclease free water (NFW). PCR amplifications were performed with following cycling conditions: (i) initial denaturation step of 5 minutes at 94°C, (ii) 30 cycles of PCR, with each cycle consisting of 30 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C, and (iii) a final extension step of 5 minutes at 72°C. PCR end products will be analysed on 1.2% agarose gel, stained with ethidium bromide. Statistical analysis was performed where categorical variables was compared using the Chi-square test (or Fisherís exact test).

Detection of gyr A gene: Detection of gyr A gene Performed using primer listed in **(table 1)**. Genomic DNA extraction from *A. baumannii*was performed using the protocol described by Pu et al [10]. ThePCR were carried out in 25 µL reaction volume consisted of 5 µL of extracted DNA,12 µL PCR Master mix, 0.3 µL(50pmol) of each primer, 7.4µl of Nuclease free water (NFW).

Amplification of DNA gyrase A subunit (gyrA) was performed according to the protocol described by de la Fuente et al [9]with following cycling conditions: 3 min at 96°C, followed by 24 cycles of 15 sec at 96°C, 30 sec at 50°C, and 90 sec at 70°C, and a final extension of 5 min at 70°C.PCR end products was analysed on 1% agarose gel, stained with ethidium bromide (0.5 mg/L) and subsequent visualization in a UV light transilluminator [9].*A. baumannii* ATCC 19606was used as reference strains for quality control.

Statistical analysis: The frequency of parameters for *A. baumannii* biofilm forming and bap gene, gyrA gene were determined in clinical samples. To compare categorical variables, chi-square or Fisher's exact test were performed. The association between biofilm formation ability and frequency of bap gene and gyrA gene with MDR *A. baumannii* was evaluated by Chi-square test. P values <0.05 were considered statistically significant.

Ethics -Ethical clearance has been obtained from institutional ethics committee of BANGALORE MEDICAL COLLEGE AND RESEARCH INSTITUTE BANGALORE.

RESULTS

Antimicrobial susceptibility testing: *Acinetobacter baumannii* isolates were predominantly found in Endotracheal aspirates. A total of 82.5% (41/50)isolates wereresistant to three or more antibiotic classes and considered asMDR organisms.Presenting a pattern of greater than 80% (40/50) resistance to fluoroquinolones (Ciprofloxacin & Levofloxacin), piperacillin/tazobactam, Cephalosporins (Ceftriaxone& Ceftazidime), followed by 75 % (38/50) resistance to Imipenem and Amikacin**(Figure 3).**

Biofilm production: The clinical isolates screened for biofilm production by qualitative tube method[2] were able to form varying degrees of biofilm.(39/50)isolates (78%)formed biofilms. Most of the isolates showed thick blue ring at the liquid-air interface.(24/39) isolates (61.5%) were strong biofilm formers while the remaining isolates were either weakly adherent (9) or non-biofilm producers (6).The overall percentage of resistance observed among all the *A. baumannii* isolates which includes biofilm producers and biofilm non producers is given in (Table 4).

The frequency of biofilm related gene and fluoroquinolone resistant gene: Presence of bap gene and gyr A gene was confirmed by detection of DNA fragment of the size 184 bp and 343 bp**(Figure 4 & 5).** 36 isolates (72%) showed the presence of bap gene of which 5 isolates were negative for biofilm producers and all the 36 isolates were multi drug resistant. 30 isolates (60%) showed presence of gyr A gene of which 3 isolates were negative for biofilm producers and all were multi drug resistant.

Their correlation with biofilm formation is shown in (Table 2& 3). Statistical analysis revealed a significant correlation between frequency of gyr A gene and biofilm formation (p=0.017) and significant correlation between bap gene and biofilm formation (p<0.001).

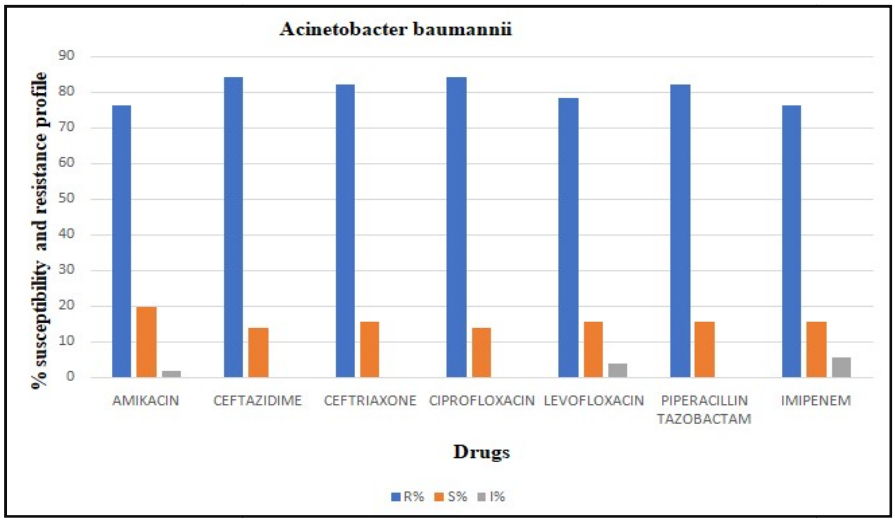


Figure 3. Antimicrobial resistance pattern of *A. baumannii* to different antibiotics

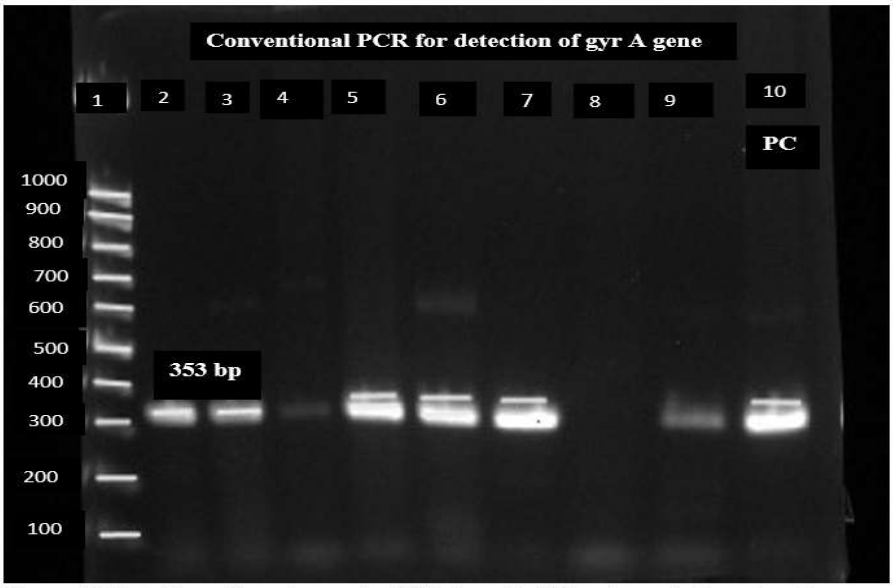


Figure 4: Detection of gyr A gene by PCR, Lane 1: Molecular weight marker, Lane 2,3,5,6,7,9 positive *A. baumannii* isolates for gyr A gene, Lane 10: *Acinetobacter baumannii* ATCC 19606.

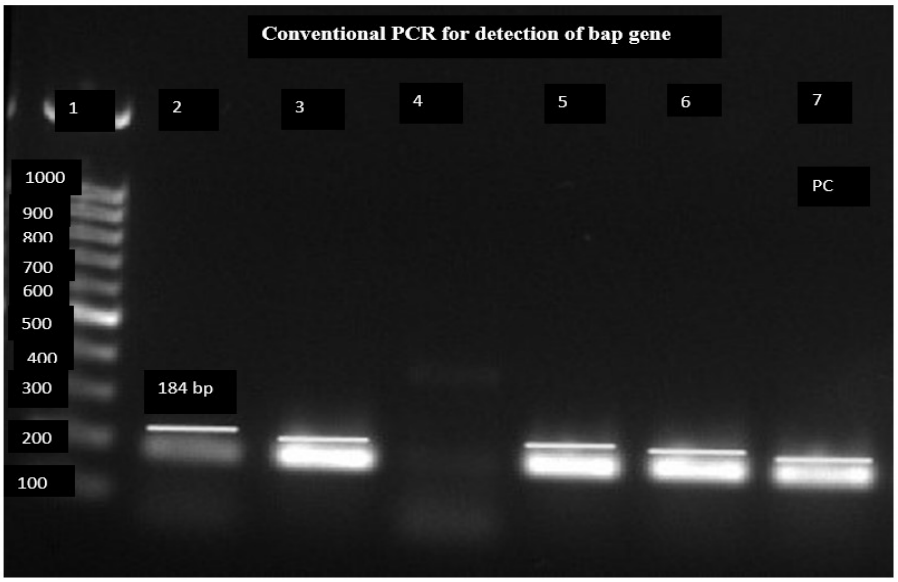


Figure 5: Detection of bap gene by PCR, Lane 1: Molecular weight marker, Lane 2,3,5,6 positive *A. baumannii* isolates for bap gene, Lane 7: *Acinetobacter baumannii* ATCC 19606.

DISCUSSION

A. baumannii thrives in hospital environments, where it can persist on surfaces, medical equipment's and in water reservoirs. Its ability to survive in such diverse conditions contributes to its prevalence in healthcare settings. It spreads through direct contact with contaminated surfaces or medical devices, as well as through the hands of healthcare workers. One of the most concerning aspects is its propensity to develop resistance to multiple antibiotics. MDR strains are resistant to most first-line antimicrobial agents, leaving clinicians with limited treatment options. leading to increased mortality, morbidity and healthcare costs. Antimicrobial stewardship programs are essential to optimize antibiotic use and minimize the development of further resistance. It is also among the most common causes of device related healthcare associated infection that results when the organism is able to resist physical and chemical disinfection, often by formation of biofilm [2]. Its thought to be a key pathogenic feature especially in relation to intravascular line infections and VAP due to its ability to adhere to surfaces and resist antibiotics. The bap gene plays a crucial role in biofilm formation this protein facilitates adherence to both biotic and abiotic surfaces, contributing to the persistence of the bacterium in clinical environments.

Furthermore, the gyrA gene is often associated with resistance to fluoroquinolone antibiotics in *A. baumannii* reducing the effectiveness and contributing to the MDR phenotype observed in many clinical isolates. We chose to investigate biofilm formation by clinical isolates of *A. baumannii* and multiple drug resistance among them and tried to correlate them in order to understand how this pathogen persist in hospital environment and cause outbreaks. In our study among 70 positive cultures collected from patients' endotracheal aspirate 50 (71%) *A. baumannii* were isolated it is the most common organism isolated from the respiratory tract and our result is in accordance with previous studies [2,3,4] and greater than 80% isolates were resistant to a wide range of antibiotic groups includes cephalosporins, fluoroquinolones, aminoglycosides and carbapenems (Figure 2). R Srinivas et al [2] found maximum resistance to imipenem (100%), cephotaxime (88%), amikacin (82%). Maria Guadalupe Avila Novoa et all [3] found (100%) resistance to piperacillin tazobactam, (60%) to amikacin, (50%) ciprofloxacin, (40%) imipenem, (73.3%) gyrA gene detected by PCR of the *A. baumannii* isolates, on CRA 73.3% isolates were biofilm producers. Bardbari A M et all [4] found (82.4%) bap gene detected by PCR and significant correlation with biofilm formation with (p= 0.004).The present results indicate the importance of adhering to the infection control measures and implementing effective antimicrobial stewardship programs to limit the dissemination of biofilm-related infections of MDR A. baumannii clones, particularly in critical hospital units.

CONCLUSION

The present study demonstrated a high propensity among clinical isolates of *A. baumannii* to form biofilm and there was a significant association of biofilm with multidrug resistance (Table 4), which is a major cause of HAIs, posing considerable challenges for infection prevention and patient management in healthcare settings.

Addressing this threat requires a comprehensive approach involving infection control measures, antimicrobial stewardship and ongoing research into new treatment strategies.

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Conflict of interest: None of the authors have any conflicts of interest to this article.

Abbreviations

A. baumannii- Acinetobacter baumannii
ATCC- American type culture collection
BAP – Biofilm associated protein
BP – Base pairs
BMCRI – Bangalore Medical College and Research Institute
CLSI- Clinical and Laboratory Standards Institute
CRA - Congo red agar
DNA- Deoxyribonucleic acid
ET – Endotracheal
GYR A- DNA gyrase A subunit
ICU – Intensive care unit
MDR- Multidrug resistant
MHA- Mueller Hinton agar
NFW – Nuclease free water
PCR – Polymerase chain reaction
PBS – Phosphate buffer saline
REF- Reference
VAP – Ventilator associated pneumonia

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