International Journal of Research in MEDICAL SCIENCE

ISSN Print: 2664-8733
ISSN Online: 2664-8741
Impact Factor: RJIF 8
IJRMS 2023; 5(1): 18-24
www.medicalpaper.net
Received: 28-03-2022
Accepted: 05-05-2022

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Investigating the biofilm trait in the clinical isolates of *Acinetobacter baumannii* from a tertiary care hospital in India

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DOI: https://doi.org/10.33545/26648733.2023.v5.i1a.42

Abstract
Background: The multidrug resistant nature and biofilm forming capacity of *Acinetobacter baumannii* have made it difficult for the clinicians and healthcare providers to treat and control its spread, leading to mortalities of 23% for hospitalized patients and 43% for patients under intensive care. Biofilm has become an important virulent factor for *Acinetobacter baumannii* as it not only protects the pathogen against the antibiotics but also enables it to evade the immune system of the host. Resistance gene transfer happens among the species and across other species in the biofilm. The relationship between biofilm trait and multidrug resistance in the clinical isolates of *A. baumannii* was investigated in this study.
Methods: Antimicrobial drug sensitivity testing was performed on the 72 *A. baumannii* isolates against two front line antibiotics: Imipenem (10 µg) and Meropenem (10 µg) by Kirby-Bauer disk diffusion method. Biofilm formation was estimated quantitatively in 96-well polystyrene plate. The biofilms were examined under scanning electron microscope (SEM) while some key regulatory genes of the biofilm synthetic pathway were screened by PCR to corroborate the phenotype.
Results: The 72 clinical isolates were characterized for carbapenem sensitivity. They were grouped as Carbapenem Resistant (CRAB) (n=58) and Carbapenem Sensitive (CSAB) (n=14) isolates. All isolates formed biofilm in our study and based on the amount of biofilm formed they were grouped as strong biofilm producers 83% (60/72), moderate biofilm producers 11% (08) and weak biofilm producers 6% (04). Certain key regulatory genes like *ata*, *bap* and *csu* were detected in almost all the isolates and the biofilms were multi-layered, closely knit and clearly visible under SEM.
Conclusions: All the isolates efficiently formed robust biofilms of which 83% of the isolates formed strong biofilms (p value <0.05). Both CRAB and CSAB formed biofilms inferring that biofilms production is inherent trait and phenotype of these clinical isolates of *A. baumannii*. The genotyping confirmed the presence of certain key genes of biofilm pathway and the strong integrity of the biofilms were revealed under SEM. Our study endorses the view that biofilms constitute an inherent trait and apparently enable *A. baumannii* to acquire MDR.

Keywords: Biofilm, *Acinetobacter baumannii*, antibiotic resistance, virulence

Introduction
Acinetobacter baumannii is a major cause for hospital acquired infections especially among the critically ill immune compromised patients [1, 2]. patients who are under special medical care like catheters, cardiac pacemakers, joint prosthesis, dentures, prosthetic heart valves and contact lenses are at great risk of getting biofilm forming bacterial infections. *Acinetobacter baumannii* is considered "critical," in the priority list of pathogens according to WHO, thus emphasizing the importance of this bacterium and its diseases. This warning is mainly because *A. baumannii* has become resistant to the "last resort" of antibiotics, the carbapenems [3, 4]. Multidrug resistant *Acinetobacter baumannii* is a serious healthcare threat. *Acinetobacter* causes a wide range of infections mostly acquired in clinical settings and is frequently associated with high morbidity and mortality rates (26–60%) [5, 6]. The rate of mortality due to multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *A. baumannii* infections is high and several outbreaks have been reported worldwide [7, 8]. The Center for Disease Control has reported that carbapenem-resistant *A.*

baumannii (CRAB) causes more than 8,500 infections, with very few treatment options left in the current scenario. Hence it is designated as a pathogen of urgent concern and a priority for novel antimicrobial development [9]. It is hypothesized that biofilms are, at least in part, responsible for the high prevalence of *A. baumannii* nosocomial and recurrent infections. Biofilms facilitate contaminating hospital surfaces and patient's indwelling devices; they protect the bacteria from the hostile antimicrobials, host's immune system. Further, they facilitate resistance genes transfer among the species and across other species. Therefore, enormous attention has been paid to understand the biofilm formation, maturation, and dispersal [10]. Biofilm formation is an important and inherent feature of most clinical isolates of *Acinetobacter* spp. [11]. Once the bacteria adhere to the surface, they transition into a biofilm state. Biofilm forms as a result of environmental signal sensing and signal transduction which, are followed by downstream cellular responses [12]. In light of the ability of *Acinetobacter* species to adhere to surfaces, form biofilms, display antibiotic resistance, and transfer genes, it has become urgently necessary to investigate these processes. Though nosocomial infections and diseases due to *A. baumannii* have been reported from India, specific studies to determine and demonstrate the biofilm trait and formation by the clinical isolates have not been reported so far. Hence, in the present study, biofilm forming potential of clinical isolates of *Acinetobacter baumannii* obtained from various specimens such as blood, urine, pus swabs, catheters, etc. was investigated. The relationship between biofilm development and multidrug resistance was investigated in this study.

Methods

Clinical isolates source

Clinical isolates of *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex were kindly provided by the Department of Clinical Microbiology of Gleneagles Global hospital, Hyderabad (n=72). They were initially isolated from different clinical specimens, which included respiratory secretions, blood, wound swabs, sputum, body fluids including urine and aspirated fluids. *Acinetobacter baumannii* ATCC 19606 was used as the reference strain.

Bacterial culture media

The *A. baumannii* isolates were grown at 37 °C on agar plates of Mueller Hinton Agar (MHA) media, Himedia Laboratories Pvt Ltd, India, Cat. No. SM173 (HM infusion B from Beef infusion 300 g/L, Acicase Casein acid hydrolysate 17.5 g/L, Starch 1.5 g/L, Agar 17.0 g/L, final pH at 25 °C of 7.3±0.1). 38.0 g of the dehydrated culture media was suspended in 100 mL distilled water in a flask. The media was heated to boiling to dissolve the contents completely, sterilized by autoclaving at 15 lbs. pressure and 121 °C) for 15 minutes. The media was cooled to 40–45 °C. It was mixed well and poured into sterile petri plates. The clinical isolates were inoculated onto Mueller Hinton Agar (MHA) and incubated overnight at 37 °C. A single, isolated pure colony was picked off the plate using a sterile inoculating loop and transferred to a 5mL of sterile Mueller-Hinton Broth (MHB) in a Falcon tube. The tubes were incubated at 37 °C for 16 h in a shaker incubator at 150 rpm. The cultures were diluted to 1.0 McFarland (A_{600nm} = 0.2 - 0.4) for all the assays.

Antimicrobial drug sensitivity testing by disc diffusion method

Antimicrobial drug sensitivity testing (AST) was performed on the *A. baumannii* isolates against two antibiotics: Imipenem (10 µg) and Meropenem (10 µg) (Himedia, Mumbai, India) as per BSAC guidelines (2015) [13] by Kirby-Bauer disk diffusion method. A single isolated colony of *A. baumannii* was inoculated into 3 mL of Mueller Hinton broth (MHB) and incubated at 37 °C over-night with shaking at 150 rpm and the turbidity was adjusted to 1.0 McFarland. A sterile cotton swab dipped in the broth culture was used to prepare a uniform lawn of *A. baumannii* on Mueller Hinton agar (MHA) plate. Imipenem (10 µg) and Meropenem (10 µg) discs were placed in the center of the freshly prepared lawn of cells and incubated at 37 °C for 24 hours. The diameter of the zone of inhibition of growth produced by the antibiotic discs was measured. The results were interpreted according to the BSAC guidelines (2015) and the *A. baumannii* isolates were classified as Carbapenem Resistant (CRAB) and Carbapenem Sensitive (CSAB) based on the growth inhibition zone diameters.

Quantitation of biofilm by Microtiter plate method

Biofilm was estimated in 96-well polystyrene plate by the method of Stepanovic *et al*. [14] with some modifications. Briefly, 200 µL of Mueller Hinton broth was added to triplicate wells of sterile 96-well polystyrene plate followed by the addition of 2 µL of bacterial culture that was grown at 37 °C overnight with shaking at 150 rpm. Negative control wells contained un-inoculated sterile plain broth. The plate was covered and incubated without shaking at 37 °C for 18 hours. Following incubation, the contents of each well was aspirated carefully, and the wells were washed three times carefully with 250 µL of Phosphate Buffer Saline (PBS). The unattached cells were removed by aspiration with pipette and the adherent bacteria were fixed with 200 µL of chilled methanol. After 15 minutes, the plate was emptied and allowed to air dry. The wells of the plate were then stained with 200 µL of 2% crystal violet. The excess dye was removed by washing the plate under running water. The plates were air dried, and the dye bound to the biofilms was extracted with 200 µL of 33% v/v glacial acetic acid and the absorbance was read spectrophotometrically at 610 nm. For each isolate the assay was run in triplicate micro-wells and in three independent experiments, the mean and standard deviations were calculated from these results using the unpaired 't' test. The 't' tests were performed by Graph Pad^{TM}. P values of < 0.05 were considered significant. Based on the absorbance, the isolates were classified into three categories: Strong biofilm producers, moderate biofilm producers and weak biofilm producers based on the following criteria:

ODC<OD≤2XODC = Weak biofilm producer
2XODC<OD≤4XODC = Moderate biofilm producer
4XODC<OD = Strong biofilm producer

Biofilm genotyping by PCR

Genomic DNA was prepared as described earlier [15] Three key regulatory genes involved in biofilms namely, *ata*, *csu* and *bap*, were studied. The primers and PCR conditions

used for screening of the biofilm genes are described in Table 1 [16, 17].

PCR assay was performed with the genomic DNA in 20 µl total reaction volume. The PCR reaction mixture contained 20pmol of each primer, 200µmol of dNTPs, 1U of Taq DNA polymerase (KAPA Taq DNA Polymerase, KAPA Biosystems Inc), 2 µl 10X buffer, 1.65 mM $MgCl_2$ and 100 ng of genomic DNA lysate. The biofilm related genes namely *ata* (a surface protein adhesin designated as *Acinetobacter* trimeric autotransporter), *csu* (csu A/B chaperone usher pili) and *bap* gene produced PCR products of sizes 110 bp, 204 bp and 220 bp respectively. The amplicons were resolved and analyzed on 2% agarose gel after ethidium bromide staining and documented in a gel documentation system (UVITEC, UK).

Scanning Electron Microscopy of biofilms

Overnight culture of *A. baumannii* isolates were adjusted to 1.0 McFarland. Cultures of *A. baumannii* were seeded on glass coverslip (10 mm X 10 mm) immersed in the wells of a 6-well microtiter plate and allowed to form biofilms. After 24 h of incubation at 37 °C, the cover slips were rinsed with distilled water to remove planktonic cells and processed for scanning electron microscopy (SEM) examination as described by Kong C [18] and Shafiei Z. [19] The samples were fixed overnight at 4 °C in cold 2.5% (v/v) Glutaraldehyde containing 0.2 M Sodium Cacodylate Buffer (SCB pH=7.2). The coverslips were washed three times with 0.1M SCB buffer with 30 minutes interval to remove the excess fixative and incrementally dehydrated by replacing the buffer with increasing concentration (30%, 50%, 70%, 80%, 90% and 100%) of ethanol (Ethyl alcohol 100%: Hayman Group Ltd., UK F204325). The coverslips were then removed from ethanol and air dried at the room temperature (25 °C) for one day. All dried samples were mounted on aluminium stub (SPI supplies division of Structure Probe INC, USA no. 05072 –AB) with double sided adhesive tape and coated with ionic gold (300Aº) in sputter coating unit (Model: E-1010 *Hitachi Japan*) under high vacuum. The processed samples were examined under scanning electron microscope (SEM) (S3400N *Hitachi Japan)* at 15KV, and high vacuum (10^{-7}Torr) and scanned pictures were taken at 10 µm and 20 µm magnifications.

Results

Antimicrobial Sensitivity Tests (AST) profile

The 72 clinical isolates were characterized for carbapenem sensitivity and grouped as Carbapenem Resistant (CRAB) (n=58) and Carbapenem Sensitive (CSAB) (n=14). The isolates were considered CRAB if the inhibition zone diameter was ≤13mm (Imipenem) and ≤12mm (Meropenem). CSAB isolates showed an inhibition zone of ≥ 25mm (Imipenem) and ≥ 20mm (Meropenem).

Biofilm formation and quantitation using micro titre plate (MTP) method

All the 72 isolates were allowed to form biofilm in the wells of micro titre plates and the biofilm was quantitated by the MTP method. All the isolates adhered strongly to the surface of the polyvinyl micro titre plates and formed biofilms (Figure 1). The crystal violet was extracted from the stained biofilms by glacial acetic acid and its absorbance measured in a spectrophotometer at A_{610} nm The isolates were classified based on the absorbance as: strong producers (OD > 1.57 ± 0.71), moderate producers (OD 0.55 ± 0.07) and weak producers (OD 0.26 ± 0.06). All the isolates formed biofilm in our study. 83% (60) of the isolates were strong biofilm producers, 11% (08) of the isolates were moderate biofilm producers and 6% (04) of the isolates were weak biofilm producers (Figure 2).

The significance of the biofilms formed by the 72 isolates were statistically analysed using the unpaired t test. The 't' tests were performed by Graph Pad™. The analysis (Figure 3) revealed that significant number (60/72=83%) of the clinical isolates formed strong biofilms (p value <0.05) and 100% of the isolates were able to form biofilms. The MTP assay was done in triplicates and the mean and standard deviation was calculated from 6 data sets.

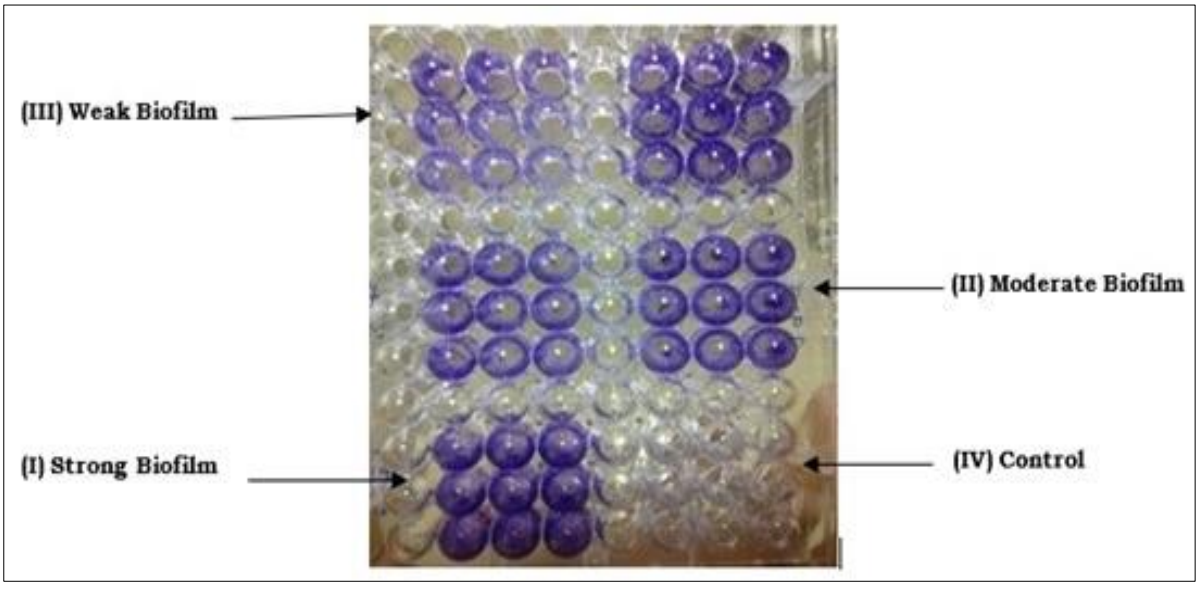

Fig 1: Quantitation of Biofilm by microtiter plate method. The adherent biofilm in each well was stained with crystal violet and the unbound dye was washed off. The crystal violet bound to the biofilms was extracted with glacial acetic acid and the absorbance of the extracted dye was measured at A_{610} nm. The intensity of the staining was also discernible visibly and the isolates were grouped based on crystal violet dye binding as (I) Strong, (II) moderate (III) weak biofilm producers and (IV) control (dye control).

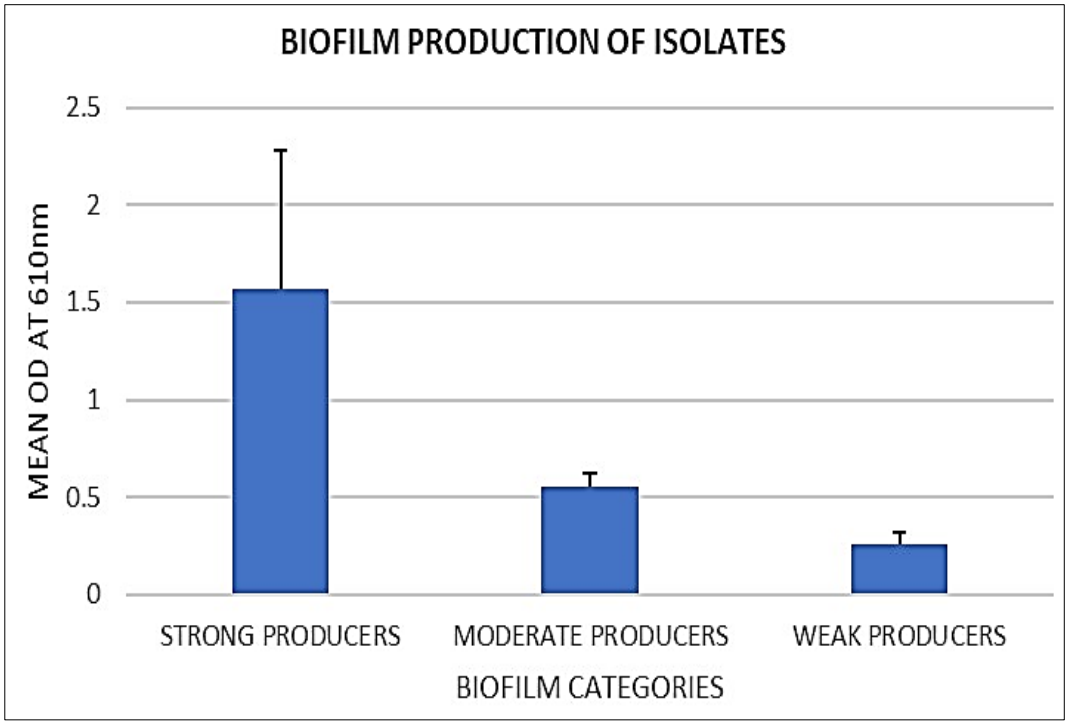

Fig 2: Bar diagram representing the biofilm profile among the clinical isolates of *A. baumannii* depicting mean biofilm OD at 610nm of strong, moderate, and weak biofilm producing isolates. The significance of the biofilms formed by the 72 isolates were statistically analysed by the unpaired 't' test (Graph Pad™). The bars represent the mean OD at 610 nm and the error bar represents the standard deviation of mean of 6 data sets.

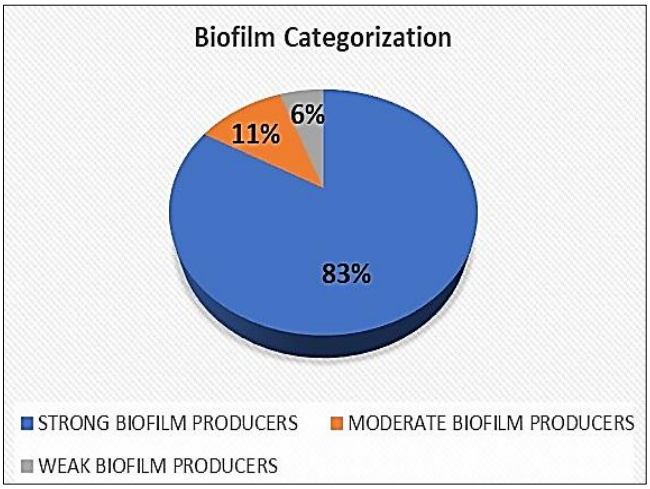

Fig 3: Biofilm profile (Pie Chart) of the clinical isolates of *A. baumannii*. Based on amount of biofilm formed, the isolates are classified as strong producers (OD 1.57 ± 0.71), moderate producers (OD 0.55 ± 0.07) and weak producers (OD 0.26 ± 0.06). 83% (60/72) of the isolates were strong biofilm producers, 11% (08/72) of the isolates were moderate biofilm producers and 6% (04/72) of the isolates were weak biofilm producers.

Genotyping by PCR to detect and confirm the presence of biofilm synthesis genes

Isolates were also screened by PCR for biofilm related genes, namely *ata* (a surface protein adhesin designated as *Acinetobacter* trimeric autotransporter), *csu* (csu A/B chaperone usher pili) and *bap* (biofilm associated protein required for development of biofilms). Of the 72 isolates, 64 (90%) isolates showed the presence of *ata* gene. *bap* gene was detected in 41 (56%) isolates and *csu* gene showed its presence in 60 (83%) isolates (Table 2). Genotyping thus confirmed the presence of active biofilm synthetic pathway and the expected gene nucleotide sequences (Figure 4).

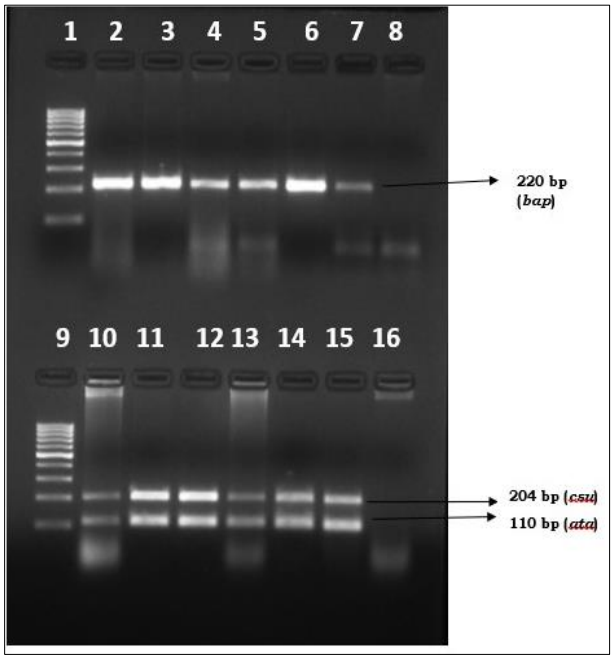

Fig 4: Demonstration of the presence of biofilm regulatory genes *bap* (220 bp), *csu* (204 bp) and *ata* (110 bp) in clinical isolates of *A. baumannii* by PCR-Agarose gel electrophoresis. Lane-1, 9: Middle range Ladder (100-1000 bp), Lane-2,3,4,5 and 7: clinical isolates, Lane-6 and 14: Positive Control, Lane-8 and 16: Negative Control

Microscopic observations of Biofilm

Under the Scanning electron microscope cells in the biofilm appeared to have regular smooth spherical cells with intact cell walls and membranes arranged very closely on a matrix (Figure 5)

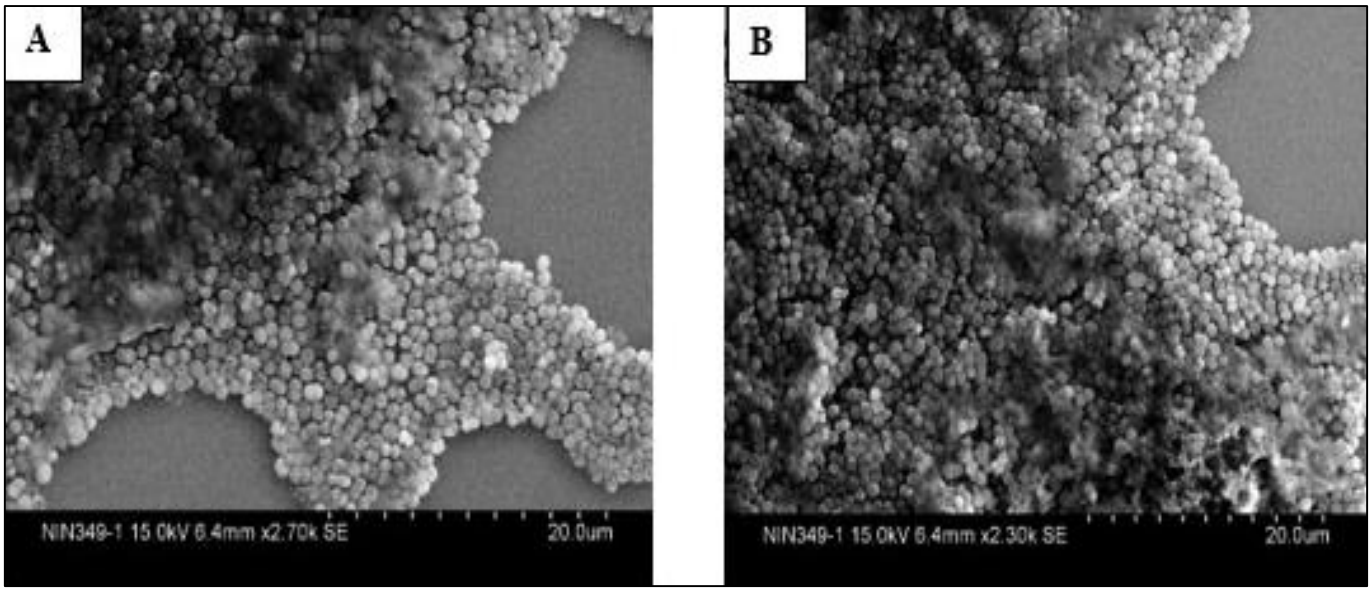

Fig 5: Scanning Electron Microscopy images of biofilm of *A. baumannii*. A: 18-hour biofilm and B: 24-hour biofilm. Biofilms were grown on glass coverslips from cultures of *A. baumannii*. After 24 h of incubation, the coverslips were rinsed with distilled water to remove planktonic cells and processed for scanning electron microscopy (SEM) examination (20 µm).

Discussion

Our study revealed that one of the possible biological reasons for innate multidrug resistance among *A. baumannii* is its ability to form biofilms. All the 72 isolates examined in this study produced biofilms. Since biofilm formation is an inherent capability, even the weak or moderate ones will show an MDR phenotype. Among the 72 isolates, 58 isolates are reported to be Carbapenem resistant and 14 isolates as Carbapenem sensitive. Since 14 out of 72 isolates turned out to be CSAB (20%), it is important to evaluate the sensitivity of all the clinical isolates to carbapenems before initiating antimicrobial therapy. It will also be a useful to initiate a large-scale screening for Carbapenem sensitivity among clinical isolates of *A. baumannii* in India. Most of the isolates recovered were carbapenem resistant indicating the high prevalence of MDR *A. baumannii* in hospital settings [20]. The relationship between biofilm development and multidrug resistance was investigated in this study. Surprisingly, all the 72 *A. baumannii* clinical isolates in this study were from immunocompromised hospitalized patients; all isolates had the ability to form biofilm, and 83% of isolates showed high biofilm-forming ability. Our results are consistent with previous reports which showed that more than 75% of *A. baumannii* isolates form biofilms [21].

The difference in the biofilm formation by the Carbapenem sensitive (CSAB) and Carbapenem resistant (CRAB) isolates was recorded. Even carbapenem sensitive isolates were capable of producing biofilms which apparently indicates that the biofilms production is an innate to *A. baumannii*. This makes it an ideal candidate to acquire multi drug resistance in hospitalized patients and may enhance colonization and persistence in the hospital environment. The AST profile of these isolates substantiates this supposition [22, 23] 88% of the isolates which formed stronger biofilms were found to be CRAB. This gives us a strong indication that the biofilms contribute to carbapenem resistance and MDR in *A. baumannii* and that there exists a positive relationship between biofilm formation and antibiotic resistance. It is probably an additional mechanism acquired by the bacterium for its survival against antimicrobial compounds as reported by others [24].

A. baumannii, which is originally an environmental microbe, has emerged in the recent decade as an important opportunistic pathogen in hospital environment especially among immunocompromised patients [25]. Successful outcome of antimicrobial therapy depends on knowing the innate mechanisms of drug resistance prevalent in the clinical isolates in a particular geographical location [26]. Usually the clinical microbiology laboratories report *Acinetobacter* positive samples as *Acinetobacter calcoaceticus–baumannii* complex. This complex consists of 4 to 5 individual species, each one with a distinct antimicrobial sensitivity. Therefore, AST evaluation of ACB complex may result in unpredictable antimicrobial treatment outcome. Also *A. baumannii* has 80-90% higher capability to form biofilms compared to other members of the ACB complex which have much lower capacity (5-24%).[27] Hence it is also important that pure cultures of *A. baumannii* be obtained and tested for AST and evaluating the biofilm potential of these isolates will certainly enable in choosing the appropriate antibiotic(s).

Table 1: List of primers and PCR conditions used in Biofilm genes screening

Sl.No	Primer Sequence (5'-3')	Amplicon size	PCR conditions	Reference
1	***abaI* F:** AATGCCTATTCCCTGCTCAC ***abaI* R:** ATTGCTTCTTGCAGAATTGC	132 bp	95°C - 5min 95°C - 30s 55°C - 40s 72°C – 60s (x35) 72°C – 10m	15
2	***ataI* F:** ATTCGGTGCTGTTGCACAAG	110 bp	95°C - 5min	16

	***ataI* R:** CACCCGGTTTATTACCAGAG		95°C - 30s 55°C - 40s 72°C – 60s 72°C – 10m (x35)	
3	***bap* F:** GTACTCCAGCAACGGTTGTA ***bap* R:** GAAGGATCTGCTGTATTCCA	220 bp	95°C - 5min 95°C - 30s 55°C - 40s 72°C – 60s 72°C – 10m (x35)	17
4	***csu* F:** ATGCGGTAAATACTCAAGCA ***csu* R:** TCACAGAAATATTGCCACCT	204 bp	95°C - 5min 95°C - 30s 55°C - 40s 72°C – 60s 72°C – 10m (x35)	17

Table 2: Prevalence of biofilm related genes in *A. baumannii* clinical isolates

S. No.	Biofilm gene	No. of isolates positive (n=72)	Positive %
1.	*ata*	64	90
2.	*bap*	41	56
3.	*csu*	60	83

Acknowledgements
This work was supported by Global Medical Education and Research Foundation (GMERF) Hyderabad. We thank Department of Microbiology, Gleneagles Global Hospital (Dr. Ranganathan Iyer) for providing the clinical isolates.

Declarations
Funding: No external funding was received. The study was supported by internal accruals.

Conflict of interest: None

Ethical approval: Not required.

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