Original Research Article

OmpA and Bap genes as Virulence Genes Involved in Biofilm Formation of Acinetobacter Baumannii

Abstract

Background: There are a variety of virulence genes involved in biofilm formation of Acinetobacter Baumannii (A. baumannii) like ompA and bap genes. Analysis of these genes can provide important data to confirm their role in biofilm formation or antibiotic resistance phenotypes. The aim of this work was to study virulence genes involved in biofilm formation of A. baumannii to be taken in consideration in prophylactic approaches, treatment, and infection control program.

Methods: The prospective study was carried out on 100 isolates including (sputum, endotracheal aspirate, pus from open wounds or open abscesses, mid-stream urine from non-catheterized patients and catheter-stream urine in catheterized patients, blood samples and body fluids) were collected from different ICUs. Isolates were identified and antibiotic susceptibility testing were done for 21 antibiotics by BD Phoenix Automated System. Biofilm formation was measured by microtiter plate for all isolates, then conventional PCR was done for detection of ompA and bap genes.

Results: The comparison of biofilm strength and MDR showed significant differences among different groups ($P<0.05$). The presence or absence of OmpA and bap genes was associated with biofilm biomass (with a P value <0.001). Among the 100 A. baumannii isolates, both genes were present in 44 isolates (44%). The strains having both OmpA and Bap genes (27/27) form stronger biofilms (100%) than isolates carrying one gene only ($P <0.001$). The association between the presence of virulence genes and MDR status was evaluated. The genes encoding OmpA were present at a higher frequency in MDRAB than in non-MDRAB strains ($P=0.014$). Bap gene was present in 48/90 (53.3%) MDRAB isolates versus only 2/10 (20%) of non-MDRAB strains ($P=0.046$).

Conclusions: There is a significant association between MDR and the biofilm-forming ability of A. baumannii. Biofilm related genes (ompA and bap) influence the intensity of the formed biofilms and were associated with multidrug-resistant A. baumannii strains.

Keywords: Virulence, Genes, Biofilm, Formation, Acinetobacter Baumannii.

Introduction:

Health care associated infections (HAI) are one of the leading causes of death and a major source of morbidity in acute care hospitals. Part of this morbidity and mortality is due to increased antibiotic resistance in HAI, which renders standard treatment ineffective. It has been estimated that more than 70% of bacteria that causes HAI are resistant to at least one antibiotic commonly used in treatment [1].

Acinetobacter baumannii (A. baumannii) is a Gram-negative bacterium that causes a variety of diseases. It exists especially in health care settings such as hospital environments and has become an increasingly problem in temperate climates. Their emergence is likely related in part to their survival ability and their rapid development of resistance to the major antibiotics [2].

Extensive use of antimicrobial chemotherapy within hospitals has contributed to the emergence and increase in the number of A. baumannii strains that are resistant to a wide range of antibiotics including broad-spectrum B-lactams, aminoglycosides, and fluoroquinolones [3]. A. baumannii is now among the most important nosocomial pathogens and is isolated frequently particularly in ICU settings where it is a cause of serious infections such as ventilator-associated pneumonia, secondary meningitis, urinary tract infection, wound, skin and bloodstream infections. It affects mainly severely debilitated patients, and it is often associated with high morbidity and mortality rates [4].

Treatment of infections caused by epidemic strains of A. baumannii are often extremely difficult because of the widespread resistance of strains to diverse antimicrobial agents, and also because this microorganism easily spreads and persists in hospital settings, that favoring transmission between patients, either via human reservoirs or via inanimate materials [5].

The A. baumannii MDR phenotype seems to play an important role in the remarkable capacity of the microorganism to persist and spread in the hospital environment, together with its ability to colonize both biotic and abiotic surfaces and to grow as biofilm [6].

Biofilms are sessile bacterial communities enclosed in a matrix comprised of extracellular material that can include polysaccharide, protein, and DNA. Biofilm formation by bacterial pathogens is associated with enhanced tolerance to host immune defenses, disinfectants, and antimicrobials [7].

It is becoming evident that biofilm-forming ability can be considered one of the main virulence factors common to a large number of A. baumannii clinical isolates [8].

This ability of A. baumannii to grow as biofilm on abiotic surfaces plays an important role in causing nosocomial infections, due to the surface colonization of hospital equipment and indwelling medical devices, such as urinary catheters, central venous catheters, endotracheal tubes, etc. [9].

There are a variety of virulence determinants involved in biofilm formation of A. baumannii. Analysis of virulence genes can provide good data to confirm their association with biofilm or antibiotic resistance phenotypes [10].

There is a strong correlation between A. baumannii ability to adhere and form biofilm, propensity to cause outbreaks and life-threatening infections and increasing antibiotic resistance [11].

The aim of this work was to study virulence genes involved in biofilm formation of Acinetobacter baumannii to be taken in consideration in prophylactic approaches, treatment, and infection control program.

Materials and Methods:

This study was carried out on 100 samples positive for A. baumannii, collected from patients admitted to ICUs of Internal medicine, chest, neurology, cardiology, emergency and anesthesia departments of Tanta University Hospitals. The study was done at Clinical Pathology Department over a period of one year, from September 2018 to 2019. The study was performed after being approved from the institutional ethical committee, Tanta University. Informed written consent was obtained from all patients included in the study.

Inclusion criteria were patients suffering from urinary tract infection (UTI), respiratory tract infection (RTI), septicemia, wound infection with fever starting more than 3 days after admission.

Exclusion criteria were all samples with laboratory confirmed isolates other than A. baumannii, Patients showed good response to antibiotic therapy, Patient who had received antibiotics in the last 1 week before sampling, and pediatric population <18 years of age.

Collection of samples were done under strict aseptic precautions according to standard protocols and processed at once.

Full history was taken **from the selected patients and patients' records were reviewed for (**Age, Gender, Clinical diagnosis at admission, Symptoms and signs of infection, History of any long-term hospitalization and duration of hospital stay, Any previous antimicrobial therapies, Risk factors increasing acquiring infection such as [Urinary catheter, Immune suppression status e.g., steroids therapy, malignancy, diabetes mellitus, chronic pulmonary

disease, chronic liver disease or chronic renal insufficiency, and Obstruction of urinary tract e.g., stones or prostatic hypertrophy]).

Equipment for samples collection and processing: wide-necked leak-proof sterile containers and sterile cotton swabs, pipettes, syringes and calibrated loops.

Media: Nutrient agar, Blood agar, MacConkey's agar, Cysteine lactose electrolyte deficient (CLED) agar for urine samples and Trypticase soy broth (TSB) (all were available from Oxoid, UK).

Gram Stain: was done for the colonies

Identification tests and antibiotic susceptibility (ID & AST): BD Phoenix Gram Negative Combo Panel (NMIC/ID-431), Accessories included: ID Broth, AST Broth, AST Indicator and Phoenix Tips. According to manufacturer, *A. baumannii* was tested against the following antibiotics by MIC test: amikacin (AMK), gentamicin (CM), imipenem (IPM), meropenem (MEM), ceftazidime (CAZ), cefepime (CEF), ampicillin-sulbactam (SAM), piperacillin-tazobactam (TZP), trimethoprim-sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LEV), minocycline (MIN). The results were classified S, I, R according to **CLSI 2018, 2019**

Materials for detection of biofilm formation: This was determined by microtiter plate method using Microtiter plate, Trypticase soy broth (TSB) with 0.25% glucose (Oxoid, UK), Phosphate buffered saline (PBS), pH 7.2 (Himedia), 1% crystal violet (Himedia), Ethanol-acetone (80:20 v/v) and Microplate reader

Materials for Conventional PCR for virulence genes (ompA & bap):

DNA extraction kits: (QIAamp $^{(R)}$ Genomic DNA Purification Kit): Buffer AL lysis buffer, Buffer AE Elution buffer, concentrate Buffer AW2 Wash buffer 2, concentrate Buffer AW1 Wash buffer 1, Buffer ATL, Proteinase K solution, QIAamp Mini Spin Column, Other materials (1.5ml microcentrifuge tubes, Water bath, 56°C, Water bath, 70°C and 97% ethanol at room temperature)

Nucleic acid amplification kits: (DreamTaq^{TM} Green PCR Master Mix): 2 × 1.25ml Dream Taq Green PCR Master Mix, 2X and 1.25ml Nuclease-Free Water

Primers:

1- **ompA:** F (GTTAAAGGCGACGTAGACG)
   R (CCAGTGTTATCTGTGTGACC)
2- **bap:** F (ATGCCTGAGATACAAATTAT)

R (GTCAATCGTAAAGGTAACG)

Materials for agarose gel electrophoresis: 1% agarose, TBE (Tris Hcl Boric acid EDTA) buffer, Ethidium bromide (1 µg / ml in running buffer), Agarose electrophoresis unit **(Standard Power Pack P25 supply biometra) and** Ultraviolet trans-illuminator (Fisher, USA).

Methods:

1- Samples collection and transport [12]:

All samples were collected under complete aseptic precautions. The samples included (Sputum samples, Endotracheal aspirates, Bronchoalveolar lavage (BAL), Septic wound swabs, Infected burn swabs, Urine samples and Blood samples).

Samples were labeled and delivered as soon as possible to the Clinical Microbiology Lab (CML) in Clinical Pathology Department.

Sputum samples: Sputum was collected in the morning in a sterile, dry, wide-necked, leak-proof container. The patient was requested to cough deeply to produce a sputum specimen to ensure that the specimen is sputum not saliva. **Endotracheal aspirates and bronchoalveolar lavage:** Bronchoalveolar lavage, wash and endotracheal aspirate were taken by the physicians according to the standard technique and collected in a sterile sputum trap. **Pus swabs:** Pus from an abscess was collected at the time the abscess was incised and drained, or after it had ruptured naturally. When collecting pus from abscesses and wounds, special care was taken to avoid contaminating the sample with commensal organisms from the skin. As far as possible, a sample from a wound was collected before an antiseptic dressing was applied. For wounds, a sterile cotton-wool swab was used to collect a sample from the infected site. **Urine samples:** In non-catheterized patients: Morning mid-stream urine was collected in sterile, dry, wide mouth and leak proof with a tightly fitted lid container after informing the patients to clean the area around the urethral opening with clean water and soap. In catheterized patients: samples were collected under complete aseptic conditions using a sterile syringe from distal end of urinary catheter after disinfection with 70% ethyl alcohol. **Blood samples:** Ten ml of venous blood were withdrawn from each patient under strict aseptic conditions and then inserted through the rubber line of the blood culture bottles. All bottles were incubated at 35°C for up to 7 days, under aerobic condition and sub-cultured every 48 hours.

2- Processing of specimens: All collected samples were processed in the Clinical Microbiology Lab (CML) in Clinical Pathology Department, Tanta faculty of medicine.

Isolation and identification of the infecting organism: All samples were cultured on MacConkey, nutrient agar and blood agar plates and then Gram stain smears were made after the culture to avoid the contamination of the sample and examined microscopically. Urine samples were cultured also on CLED agar by inoculating a loopful 1µl of well mixed uncentrifuged urine using a sterile calibrated bacteriological loop by surface streak method. All plates were incubated at 37°C for 24 h and then the isolates in the primary plates were identified by: **Colonial morphology:** Size, shape, surface and color of the colony, characteristic feature of the growth e.g., pigment production. For urine samples on CLED agar: colonies were counted. The number of colonies obtained was multiplied by 1000 to obtain the CFU/ml. The significant colony count is a pure growth of $\geq 10^5$ CFU/ml in case of bacteriuria in non-catheterized patients and $\geq 10^3$ CFU/ml in catheterized patient. **Microscopic examination:** Gram-stained film was prepared and examined.

Identification of *Acinetobacter baumannii* was done by: Gram-stained film: Gram negative cocco-bacilli. Cultural characters: study of colonial morphology: On MacConkey agar plates: Lactose non-fermenting (pale yellow) colonies. Colonies from CLED plates were characterized by Lactose non-fermenting (yellow) colonies. After colonial description, pure colonies were subjected to gram stain (Secondary gram stain). *A. baumannii* was identified by Phoenix BD with NMIC-431 panels (Becton Dickinson, USA).

Antibiotic susceptibility testing: All the tests were performed according to manufacturer's instructions. Phoenix BD with NMIC-431 panels (Becton Dickinson, USA): dedicated AST indicator and 25 µl of bacteria suspension were added into Mueller-Hinton broth (MHB). These suspensions were subsequently added to panels, which were placed in Phoenix BD instrument, the results with their interpretation were obtained automatically after less than 18h according to**: (CLSI 2018, 2019)** The confirmed *Acinetobacter baumannii* subspecies isolates were preserved in TSB at -20°C for further testing by conventional PCR to detect virulence genes (OmpA, Bap).

3- Biofilm Formation: Each isolate was grown overnight in trypticase soy broth (TSB) with 0.25% glucose at 37°C. The overnight growth was diluted in a ratio of 1:40 in TSB-0.25 % glucose. Two hundred microliter of cell suspension was inoculated in sterile 96 well polystyrene microtiter plates. After 24 h of incubation, the wells were gently washed three times with 200 microliter of phosphate buffered saline (PBS) then dried in an inverted position and stained with 1% crystal violet for 15 min. The wells were rinsed again in 200 microliters of ethanol-acetone (80:20

v/v) to solubilize crystal violet. The optical density at 620 nm (OD_{620}) was determined using microplate reader. Each assay was performed in triplicate and the average optical density was considered.

The following values were assigned for biofilm determination: non-biofilm producer: $OD_{620} < 0.275$, Weak biofilm producer: $0.275 \leq OD_{620} < 0.55$, Medium biofilm producer: $0.55 \leq OD_{620} < 1.1$, and Strong biofilm producer: $1.1 \leq OD_{620}$. The number 0.275 was chosen for guideline because it was three standard deviations above the mean OD (0.303) of a clean microtiter plate stained by the above method.

4- Detection of two virulence genes (ompA & bap) involved in biofilm formation of *A. baumannii*: DNA Extraction Procedure for total genomic of samples according to manufacturer protocol of GeneJET Genomic DNA Purification Kit Thermo Scientific:

1- 350 µL of Lysis Buffer A were pipetted into 1.5 mL microcentrifuge tube (not provided). Bacterial pellet was weighed up to 100 mg of fresh or frozen tissue: up to 20 mg of lyophilized tissue. 100 mg of plant tissue were placed into liquid nitrogen and grind thoroughly with a mortar and pestle. 100 mg of tissue were placed into a vial containing stainless steel beads. The vial and beads should be precooled with liquid nitrogen. The setup of the mechanical disruption depends on the tissue type.
2- 50 µL of Lysis Buffer B were added and 20 µL RNase A.
3- The sample was incubated 10 min at 65°C vortexing occasionally or use a shaking water bath, rocking platform or thermomixer.
4- 130 µL of Precipitation Solution were added and were mixed by inverting the tube 2-3 times. The tube was incubated 5 min on ice.
5- Then, it was centrifuged for 5 min at ≥20,000 × g (≥14,000 rpm).
6- The supernatant (usually 450-550 µL) was collected and transferred to the clean microcentrifuge tube (not provided). 400 µL of Plant gDNA Binding Solution and 400 µL of 96% ethanol were added and were mixed well.
7- Half of the prepared mixture (600-700 µL) were transferred to the spin column. It was centrifuged for 1 min at 6,000 × g (~8,000 rpm). The flow-through solution was discarded, and the remaining mixture was applied onto the same column. Then, it was centrifuged for 1 min at 6,000 × g (~8,000 rpm).
8- 500 µL of Wash Buffer I were added to the column (ensure ethanol has been added to Wash Buffer I). It was centrifuged for 1 min at 8,000 × g (~10,000 rpm). The flow through was discarded, and the column was placed back into the collection tube.

9- 500 µL of Wash Buffer II was added to the column (ensure ethanol has been added to Wash Buffer II). It was centrifuged for 3 min at maximum speed ≥20,000 × g (≥14,000 rpm). The collection tube was emptied. The purification column was placed back into the tube and the column was re-spun for 1 min. at maximum speed (≥20,000 × g, ≥14,000 rpm). The collection tube containing the flow-through solution was discarded, and the column was transferred to a sterile 1.5 mL microcentrifuge tube (not provided)

10- To elute genomic DNA,100 µL of Elution Buffer were added to the center of the column membrane, it was incubated for 5 min at room temperature and was centrifuged for 1 min at 8,000 × g (~10,000 rpm)

11- A second elution step was performed using 100 µL Elution Buffer. You may perform the second elution using the same elution tube or in a different tube. The purified DNA was ready to be used in downstream applications or stored at -20°C.

PCR amplification:

DreamTaq Green PCR Master Mix (2X) (K1081, Thermo Fisher, USA) was used for specific gene amplification according to manufacturer protocol through Creacon (Holland, Inc) Polymerase Chain Reaction (PCR) system cycler. The reaction consists of Initial denaturation step at 95 °C for 60 seconds, denaturation step 94 °C, annealing step 50 °C, extension 72 °C for 60 seconds and final extension 72 °C for 3 minutes, total of cycles 22 cycle [13].

Agarose gel electrophoresis and detection of the amplification products:

1.0 % agarose solution was prepared by adding 0.75g agarose to 50ml of 1x TBE electrophoresis buffer in 50ml flask. Heating in a microwave oven then the agarose was dissolved. The agarose was cooled in 50°C. A comb was inserted in electrophoresis bed and the agarose was poured in it.

Great care should be taken during pouring of the agarose to avoid bubbles formation. The gel solidified within 15 min and became cloudy, the electrophoresis apparatus was filled with the electrophoresis buffer and the comb was removed creating 6 or 10 wells for sample application) in the presence of DNA ladder (peqGOLD 1 kb DNA-Ladder, Peqlab, VWR) according to manufacturer protocol. Electrodes were connected to the power supply and the later was turned on. It was adjusted at 80 Volts for 100 min. The gel was removed from its bed and transferred to the gel staining tray for staining with Ethidium bromide for 30 min followed by 20 min distain in distilled water.

Data analysis:

Gel documentation system (Geldoc-it, UVP, England) was applied for data analysis using Total lab analysis software (ww.totallab.com, Ver.1.0.1). Positive amplicons with 1500 bp were eluted from agarose gel. Resultant PCR products were purified with Micro spin filters and quantities spectrophotometrically. Sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea).

Table 1: Sequences and products of OmpA and Bap genes:

Primer	Sequences	Products bp	Reference
OmpA	GTTAAAGGCGACGTAGACG CCAGTGTTATCTGTGTGACC	578	Talib et al. 2018
Bap	ATGCCTGAGATACAAATTAT GTCAATCGTAAAGGTAACG	1449	

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Kolmogorov test was used to verify the normality of distribution Quantitative data were described using range (minimum and maximum), mean, standard deviation, median and interquartile range (IQR). Significance of the obtained results was judged at the 5% level. Chi-square test was used for for categorical variables, to compare between different groups. Fisher's Exact for chi-square Correction when more than 20% of the cells have expected count less than 5. Kruskal Wallis test for abnormally distributed quantitative variables, to compare between more than two studied groups and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons. a p value < 0.05 was considered statistically significant.

Results:

Isolates from sputum showed the largest percentage (47%), followed by pus (20%), urine (15%), blood (10%), and the other specimens (obtained from peritoneal, pleural fluids, tracheal tube & bronchoalveolar lavage) showed the least percentage (8%). All *Acinetobacter baumannii* isolates were tested to biofilm formation by microtiter plate. Among all isolates, (29%) were non biofilm producers, while the majority were biofilm producers (71%). The number of weak biofilm producers was (17%), (27%) moderate biofilm producers, and (27%) strong biofilm producers. The median OD_{620} and interquartile range (IQR) value for non-

biofilm producers was 0.21 (0.11, 0.26), for weak biofilm producers, 0.39 (0.29, 0.51), moderate biofilm producers, 0.79 (0.59, 0.99) and strong biofilm producers, 1.47 (1.2, 1.6). **Table 2**

Table 2: Distribution of the studied cases according to demographic, specimen, OD620, biofilm and biofilm strength (n = 100)

	No.	**%**
Sex		
Male	55	55.0
Female	45	45.0
Age (years)	57.56 ± 11.56	
Range	29.0 – 80.0	
Specimen	No.	%
Sputum	47	47.0
Pus	20	20.0
Urine	15	15.0
Blood	10	10.0
Other	8	8.0
OD_{620} Median (IQR)	0.65 (0.24 - 1.25)	
Biofilm	No.	%
Negative	29	29.0
Positive	71	71.0
Biofilm strength	No.	%
Non	29	29.0
Positive	71	71.0
Weak	17	17.0
Moderate	27	27.0
Strong	27	27.0

SD: Standard deviation, IQR: interquartile range

The comparison of biofilm biomass OD_{620} among various sources of specimens showed significant differences among different groups (P<0.05). **Table 3**

Table 3: Relation between Biofilm strength and OD620

OD620	**Biofilm strength**				**H**	**p**
	Non (n= 29)	**Weak (n= 17)**	**Moderate (n= 27)**	**Strong (n= 27)**		
Min. – Max	0.11 – 0.26	0.29 – 0.51	0.59 – 0.99	1.20 – 1.60	92.257*	<0.001*
Mean ± SD.	0.20 ± 0.04	0.40^{a} ± 0.07	0.79^{ab} ± 0.11	1.44^{abc} ± 0.11		
Median	0.21	0.39	0.79	1.47		

H: H for Kruskal Wallis test, pairwise comparison bet. each 2 groups were done using Post Hoc Test (Dunn's for multiple comparisons test), p: p value for association between different categories, a: significant with Non, b: significant with Weak, c: significant with Moderate

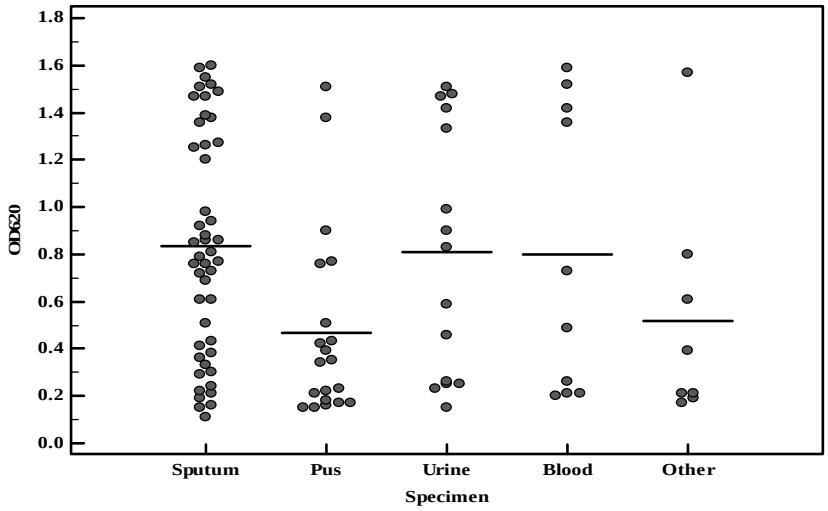

Figure 1: Relation between specimen and OD_{620}

Table 4: Relation between Biofilm strength and Specimen:

Specimen	Biofilm strength								χ^2	^{MC}p
	Non (n= 29)		Weak (n= 17)		Moderate (n= 27)		Strong (n= 27)			
	No.	%	No.	%	No.	%	No.	%		
Sputum	7	24.1	8	47.1	17	63.0	15	55.6	17.459	0.093
Pus	9	31.0	6	35.3	3	11.1	2	7.4		
Urine	5	17.2	1	5.9	4	14.8	5	18.5		
Blood	4	13.8	1	5.9	1	3.7	4	14.8		
Other	4	13.8	1	5.9	2	7.4	1	3.7		

χ^2: **Chi square test, MC: Monte Carlo,** p: p value for association between different categories

All isolates were tested for their antibiotic susceptibility toward 12 antibiotics. Most isolates were resistant to gentamicin (70%). The *A. baumannii* isolated strains were also resistant to amikacin (55%), imipenem (57%), meropenem (55%), ceftazidime (62%), cefepime (67%), ampicillin-sulbactam (56%), piperacillin-tazobactam (56%), trimethoprim/ sulfamethoxazole (59%), ciprofloxacin (69%), levofloxacin (61%) minocycline (66%). **Table 5**

Table 5: Distribution of the studied cases according to Antibiotics (n = 100)

Antibiotics	Sensitive		Resistant		Intermediate	
	No.	%	No.	%	No.	%
AMK	41	41.0	55	55.0	4	4.0
CM	21	21.0	70	70.0	9	9.0
IPM	34	34.0	57	57.0	9	9.0
MEM	32	32.0	55	55.0	13	13.0
CAZ	25	25.0	62	62.0	13	13.0
CEF	18	18.0	67	67.0	15	15.0
SAM	23	23.0	56	56.0	21	21.0
TZP	26	26.0	56	56.0	18	18.0
SXT	30	30.0	59	59.0	11	11.0
CIP	17	17.0	69	69.0	14	14.0

LEV	18	18.0	61	61.0	21	21.0
MIN	23	23.0	66	66.0	11	11.0

All isolates were defined as being multidrug resistant *A. baumannii* (MDRAB) when there was resistance to three or more antibiotic classes. The incidence of MDRAB was 90 % (90/100). Among 90 of MDRAB isolates, 68 (75.6 %) were biofilm-forming strains while 30 % (3/10) of non-MDRAB only produced biofilms (P = 0.006; Fisher's exact test). There was significant difference among the groups ($P \leq 0.05$). The comparison of biofilm strength and MDR showed significant differences among different groups (P<0.05). **Table 6**

Table 6: Distribution of the studied cases according to MDR and relation between MDR and Biofilm and Biofilm strength (n = 100)

MDR			**No.**		**%**	
Negative			10		10.0	
Positive			90		90.0	
Biofilm	**MDR**				**χ^2**	**^{FE}p**
	Negative (n= 10)		**Positive (n= 90)**			
	No.	**%**	**No.**	**%**		
Non	7	70.0	22	24.4	9.071*	0.006*
Positive	3	30.0	68	75.6		
Biofilm Strength						
Non	7	70.0	22	24.4	8.578*	0.020*
Weak	1	10.0	16	17.8		
Moderate	2	20.0	25	27.8		
Strong	0	0.0	27	30.0		

χ^2: Chi square test, MC: Monte Carlo, E: Fisher Exact, p: p value for association between different categories, *: Statistically significant at $p \leq 0.05$

The association between biofilm forming ability and individual drug resistance of *A. baumannii* was evaluated. The resistance rates of most antibiotics were found to be more in biofilm-forming than non-biofilm forming groups with a P-value ranging from 0.001 to 0.5. Of the 70 gentamicin resistant isolates, 56 (78.9%) strains were biofilm producers while only 6 of 21 (8.5%) of gentamicin susceptible strains were biofilm producers (P <0.001; Monte Carlo test). In *A. baumannii* that resistance to gentamicin had a significantly higher ability to build biofilms when compared with gentamicin sensitive groups (P < 0.001). The same significant P value (P < 0.001) was obtained with the following antibiotics: imipenem, meropenem, ceftazidime, cefepime, ampicillin-sulbactam, piperacillin-tazobactam, and trimethoprim/ sulfamethoxazole. For other antibiotics, no statistical correlation was observed. **Table 7**

Table 7: Relation between Biofilm and Antibiotics

Antibiotics		Biofilm				χ2	P
		Negative (n= 29)		Positive (n= 71)			
		No.	%	No.	%		
AMK	S	13	44.8%	28	39.4%	1.572	^{MC}p= 0.512
	R	14	48.3%	41	57.7%		
	I	2	6.9%	2	2.8%		
CM	S	15	51.7%	6	8.5%	24.790*	<0.001*
	R	14	48.3%	56	78.9%		
	I	0	.0%	9	12.7%		
IPM	S	16	55.2	18	25.4	32.390*	<0.001*
	R	5	17.2	52	73.2		
	I	8	27.6	1	1.4		
MEM	S	12	41.4	20	28.2	29.394*	<0.001*
	R	6	20.7	49	69.0		
	I	11	37.9	2	2.8		
CAZ	S	14	48.3	11	15.5	11.874*	0.003*
	R	12	41.1	50	70.4		
	I	3	10.3	10	14.1		
CEF	S	14	48.3	4	5.6	25.991*	<0.001*
	R	11	37.9	56	78.9		
	I	4	13.8	11	15.5		
SAM	S	13	44.8	10	14.1	21.091*	<0.001*
	R	6	20.7	50	70.4		
	I	10	34.5	11	15.5		
TZP	S	20	69.0	6	8.5	42.007*	<0.001*
	R	4	13.8	52	73.2		
	I	5	17.2	13	18.3		
SXT	S	17	58.6	13	18.3	21.013*	<0.001*
	R	7	24.1	52	73.2		
	I	5	17.2	6	8.5		
CIP	S	8	27.6	9	12.7	4.169	^{MC}p= 0.106
	R	16	55.2	53	74.6		
	I	5	17.2	9	12.7		
LEV	S	9	31.0	9	12.7	4.706	0.095
	R	15	51.7	46	64.8		
	I	5	17.2	16	22.5		
MIN	S	6	20.7	17	23.9	2.814	0.245
	R	22	75.9	44	62.0		
	I	1	3.4	10	14.1		

χ^2: Chi square test, C: Monte Carlo, p: p value for association between different categories, *: Statistically significant at $p \leq 0.05$

PCR was utilized to show the presence of virulence genes (OmpA and Bap genes) in all *A. baumannii* isolates. OmpA was more common 75/100 (75%), and Bap was 50/100 (50%). The presence or absence of OmpA and bap genes was associated with biofilm biomass (with a P value <0.001). **Table 8**

Table 8: Distribution of the studied cases according to OmpA and Bap gene and relation between Biofilm with OmpA gene and Bap gene (n = 100)

	No.	%
OmpA gene		
Negative	25	25.0

Positive			75		75.0	
Bap gene						
Negative			50		50.0	
Positive			50		50.0	
	Biofilm				**χ^2**	**P**
	Negative (n= 29)		**Positive (n= 71)**			
	No.	**%**	**No.**	**%**		
OmpA gene						
Negative	15	51.7	10	14.1	15.558*	<0.001*
Positive	14	48.3	61	85.9		
Bap gene						
Negative	27	93.1	29	32.4	30.355*	<0.001*
Positive	2	6.9	42	67.6		

χ^2: Chi square test, p: p value for association between different categories, *: Statistically significant at $p \leq 0.05$.

Among the 100 *A. baumannii* isolates, both genes were present in 44 isolates (44%). The strains having both OmpA and Bap genes (27/27) form stronger biofilms (100%) than isolates carrying one gene only (P <0.001). **Table 9**

Table 9: Relation between Biofilm strength with OmpA gene and Bap gene

	Biofilm strength								**χ^2**	**p**
	Non (n= 29)		**Weak (n= 17)**		**Moderate (n= 27)**		**Strong (n= 27)**			
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**		
BmpA gene and bap gene										
Negative	27	93.1	16	94.1	13	48.1	0	0.0	61.266*	<0.001*
Positive	2	6.9	1	5.9	14	51.9	27	100.0		
Sig.bet.Grps	$^{FE}p_1$=1.000, p_2<0.001*, p_3<0.001*, p_4=0.002*, p_5<0.001*, p_6<0.001*									

χ^2: Chi square test, p: p value for association between different categories, p_1: p value for association between non and Weak, p_2: p value for association between non and Moderate, p_3: p value for association between non and Strong, p_4: p value for association between weak and moderate, p_5: p value for association between weak and strong, p_6: p value for association between moderate and Strong, *: Statistically significant at $p \leq 0.05$

± **Figure 2**

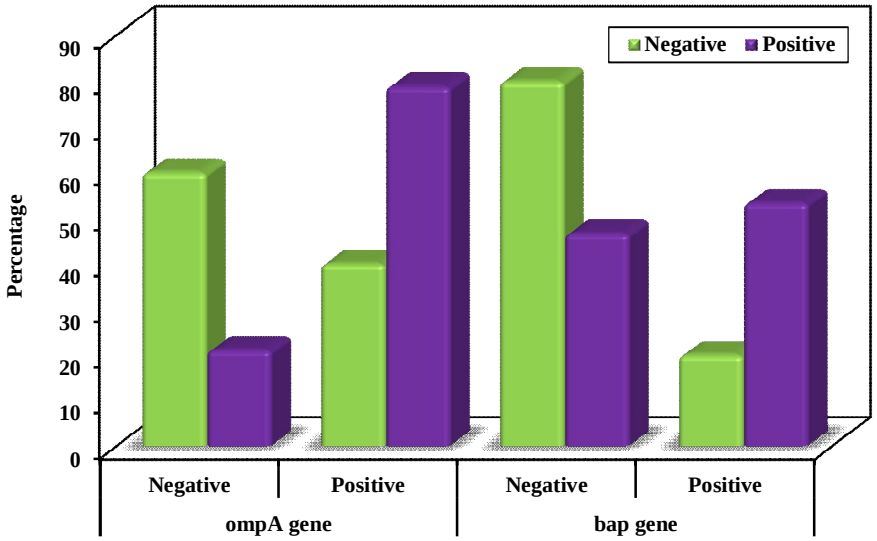

Figure 2: Relation between MDR with OmpA gene and Bap gene.

Discussion

Acinetobacter baumannii is considered a threat to public health in a global scenario mainly due to its tendency of acquiring resistance mechanisms. This feature favors its survival even under the use of selective antimicrobial agents, and therefore, disseminates multidrug-resistant strains.[14]

In the present work, we determined the biofilm formation in the clinical isolates. It was found that 71% of studied A. baumannii showed biofilm formation ability. This agreed with Thummeepak et al.[15], Ranjbar and Farahani,[16], and Chen et al. [17], who found that 76.9%, 70.6% and 54% of clinical A. baumannii isolates were positive for biofilm production respectively.

Several studies linked high frequency of biofilm formation in A. baumannii with extended survival and resistance to external stresses such as limited nutrients and dehydration.[18]

The same results were obtained in Egypt by, Asaad et al. [19], who found that 70.1% of A. baumannii clinical isolates showed biofilm formation, while Sherif et al.[20] , found that A. baumannii isolates exhibited much higher frequency 100% of biofilm production,

The comparison of biofilm biomass (OD_{620}) among the various sources of specimens. Our results were in line with Thummeepak et al. [15], who found that specimens from urine, blood, and sputum were higher to form biofilms when compared to pus and others.

Moreover, the present results were also in agreement with the results reported by Duarte et al. [21] who concluded that urine samples with A. baumannii produce biofilms with greater

biomass. They concluded that urinary tract pathogens can adhere and form biofilms specially in flowing environments, which lead to persistent infections.

Regarding the individual analysis of biofilm forming ability of different isolates. our results agreed with Zhang et al. [22], who found that most sputum specimens of A. baumannii form strong and moderate biofilm, as the bacteria attach to the host and form adherent biofilm which is responsible to its pathogenicity. This biofilm facilitates bacterial survival in different environments and leads to respiratory tract infections including health care associated pneumonia.

The high prevalence rates of Multidrug resistant Acinetobacter baumannii (MDRAB) clinical isolates have been reported globally, ranging from 21–95%.[22] Similarly, to other reports, we observed a high prevalence rate of MDRAB in our study, 90% (90/100) of our isolates were MDR displaying resistance patterns consistent with previous reports. [23, 24]

In the current study, we investigated the relationship between Multidrug resistant Acinetobacter baumannii (MDRAB) and biofilm strength, it was found that there was a significant correlation ($p < 0.05$) between biofilm strength and MDR, which agreed with Ghasemi et al.[25], who found that all strong biofilm forming A. baumannii isolates were MDR, and hence there was a significant correlation between strong biofilm formation and MDR phenotype.

In contrast, Thummeepak et al.[15], indicated that MDR phenotype had no association with biofilm producing ability of A. baumannii. While Shenkutie et al.[26], reported an inverse relationship between biofilm formation of A. baumannii and antibiotic resistance. They concluded that bacterial ability to form biofilm may be associated with antibiotic resistance at the level of the individual. A statistically significant correlation occurred with resistance to certain antibiotics.

In the present study, 78.9% of biofilm producing strains were resistant to gentamycin with p value < 0.05. This result was like the study of Thummeepak et al.[15], who found that A. baumannii strains positive for biofilm formation were more frequently resistant to gentamicin. This phenotypic correlation may be due to the presence of biofilm-associated and gentamicin resistance determinants which are co-located on the same plasmid or genomic island. Also, Duarte et al.[21], found that A. baumannii isolates resistant to gentamicin and tobramycin were more frequently able to form biofilm than susceptible strains.

In the present work, it was found that 73.2% and 69% of biofilm producing strains were resistant to imipenem and meropenem respectively. These results agreed with the results of Wasfi et al.[27], who detected resistance to meropenem and ertapenem in 70.8% of A.

baumannii isolates. This resistant profile presented by Gram-negative bacilli such as A. baumannii concerns the global public health because if there is a high isolation of strains which are resistant to carbapenems, the treatment options get more limited and will induce, therefore, the need of new therapeutic approaches, such as the use of polymyxins (colistin and polymyxin B).[21]

Several reports have notified that biofilm related genes including OmpA and Bap genes of A. baumannii were responsible for biofilm development and antibiotic resistance.[28] The present study aimed to verify the occurrence of virulence genes (OmpA, and Bap) in A. baumannii isolates and their correlation with biofilm formation and drug resistance.

Regarding OmpA gene in the present work, it was expressed in 75% of A. baumannii isolates. This was in agreement with studies done by Thummeepak et al.[15] and Zeighami et al.[29] who found that the percentages of this gene in A. baumannii isolates were 84% and 76.7 respectively.

The current results showed that 50% of A. baumannii isolates possessed Bap gene. This was in agreement with Thummeepak et al.[15], who reported that 48% and 30% of A. baumannii isolates were positive for Bap gene respectively.

In the present study, when analyzing the correlation between biofilm formation and the related virulence genes, a significant positive correlation was found between biofilm formation and the presence of either OmpA or Bap genes ($p < 0.001$). Among 71 positive biofilm producer isolates, 85.9% possessed OmpA gene, and of 29 negative biofilm producers, 51.7% did not possess OmpA gene.

For Bap gene, among 71 positive biofilm producer isolates 67.6% possessed Bap gene, and of 29 negative biofilm producers, 93.1% did not possess Bap gene.

This may represent a profound concern due to the likelihood of horizontal gene transfer between isolates closely located within biofilms.[30, 31]

These results were correlated with the results of Liou et al. and Fallah et al., who have demonstrated that biofilm associated genes, including OmpA and Bap, were responsible for the biofilm development of A. baumannii strains.

On the other hand, Thummeepak et al. [10], found that A. baumannii isolates harboring virulence genes did not promote biofilm forming ability on polystyrene, while the presence of Bap or OmpA showed an inverse correlation with biofilm formation.

Remarkably, the present study detected a statistically significant association between both OmpA and Bap genes and the biofilm-forming strength. We found that both genes were

present in 100% of strong biofilm forming bacteria, while present in 51.9% of moderate biofilm producers, and in 5.9% of weak biofilm producers.

This significance association agreed with the study of Yang et al.[32] who suggested that A. baumannii strains which carry OmpA and Bap genes, tend to produce strongly adherent biofilm when compared to isolates without these genes. Also, Al-Shamiri et al., found that these gene pairs were considered of great diagnostic value for the discrimination of strong biofilm-forming A. baumannii phenotype.

In the present study, we found a significant correlation between MDRAB strains and the presence of OmpA or Bap genes. In agreement with these results, Thummeepak et al.[10], found a strong association between MDR A. baumannii phenotype and the presence of Bap and OmpA genes. It is possible that due to OmpA being a β-barrel porin, antibiotics may be transferred from the periplasm through the outer membrane and then couples with inner membrane efflux pumps.

So, the transcriptional or translational analysis of virulence genes of A. baumannii can provide good data to confirm their association with biofilm or antibiotic resistance phenotypes which must be further analyzed.

Conclusions:

Acinetobacter baumannii is an opportunistic bacterial pathogen which is an important cause of hospital-acquired infections. Most A. baumannii strains are multidrug resistant. Antibiotic resistant rates among bacterial pathogens isolated from ICU infections represent a major problem worldwide. Biofilm producers A. baumannii strains increase the incidence of acquiring infection. There is a significant association between MDR and the biofilm-forming ability of A. baumannii. Biofilm related genes (ompA and bap) influence the intensity of the formed biofilms and were associated with multidrug-resistant A. baumannii strains.

References

1. Haque M, Sartelli M, McKimm J, Abu Bakar M. Health care-associated infections - an overview. Infect Drug Resist. 2018;11:2321-33.
2. McConnell MJ, Actis L, Pachón J. Acinetobacter baumannii: human infections, factors contributing to pathogenesis and animal models. FEMS Microbiol Rev. 2013;37:130-55.
3. Asif M, Alvi IA, Rehman SU. Insight into Acinetobacter baumannii: pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. Infect Drug Resist. 2018;11:1249-60.

4. Ayoub Moubareck C, Hammoudi Halat D. Insights into Acinetobacter baumannii: A Review of Microbiological, Virulence, and Resistance Traits in a Threatening Nosocomial Pathogen. Antibiotics (Basel). 2020;9.
5. Manchanda V, Sanchaita S, Singh N. Multidrug resistant acinetobacter. J Glob Infect Dis. 2010;2:291-304.
6. Eze EC, Chenia HY, El Zowalaty ME. Acinetobacter baumannii biofilms: effects of physicochemical factors, virulence, antibiotic resistance determinants, gene regulation, and future antimicrobial treatments. Infect Drug Resist. 2018;11:2277-99.
7. Schulze A, Mitterer F, Pombo JP, Schild S. Biofilms by bacterial human pathogens: Clinical relevance - development, composition and regulation - therapeutical strategies. Microb Cell. 2021;8:28-56.
8. Morris FC, Dexter C, Kostoulias X, Uddin MI, Peleg AY. The Mechanisms of Disease Caused by Acinetobacter baumannii. Front Microbiol. 2019;10:1601.
9. Roy S, Chowdhury G, Mukhopadhyay AK, Dutta S, Basu S. Convergence of Biofilm Formation and Antibiotic Resistance in Acinetobacter baumannii Infection. Front Med (Lausanne). 2022;9:793615.
10. Thummeepak R, Kongthai P, Leungtongkam U, Sitthisak S. Distribution of virulence genes involved in biofilm formation in multi-drug resistant Acinetobacter baumannii clinical isolates. Int Microbiol. 2016;19:121-9.
11. Sherif MM, Elkhatib WF, Khalaf WS, Elleboudy NS, Abdelaziz NA. Multidrug Resistant Acinetobacter baumannii Biofilms: Evaluation of Phenotypic-Genotypic Association and Susceptibility to Cinnamic and Gallic Acids. Front Microbiol. 2021;12:716627.
12. Baron EJ, Miller JM, Weinstein MP, Richter SS, Gilligan PH, Thomson RB, Jr., et al. A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 2013 recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM)(a). Clin Infect Dis. 2013;57:e22-e121.
13. Talib SS, Abdulrahman TR, Ali SH. Detection of Some Biofilm Genes Related with Multidrug-Resistant in Acinobacter baumannii Isolated from Clinical Isolates. Iraqi J Med Sci. 2018;16:559-64.
14. Reena AAA, Subramaniyan A, Kanungo R. Biofilm formation as a virulence factor of Acinetobacter baumannii: An emerging pathogen in critical care units. J Curr Res Sci Med. 2017;3:74-84.
15. Thummeepak R, Kongthai P, Leungtongkam U, Sitthisak S. Distribution of virulence genes involved in biofilm formation in multi-drug resistant Acinetobacter baumannii clinical isolates. Int Microbiol. 2016;19:121-9.
16. Ranjbar R, Farahani A. Study of genetic diversity, biofilm formation, and detection of Carbapenemase, MBL, ESBL, and tetracycline resistance genes in multidrug-resistant Acinetobacter baumannii isolated from burn wound infections in Iran. Antimicrob Resist Infect Control. 2019;8:172.
17. Chen L, Li H, Wen H, Zhao B, Niu Y, Mo Q, et al. Biofilm formation in Acinetobacter baumannii was inhibited by PAβN while it had no association with antibiotic resistance. Microbiologyopen. 2020;9:10-63.
18. Sung JY. Molecular characterization and antimicrobial susceptibility of biofilm-forming Acinetobacter baumannii clinical isolates from Daejeon, Korea. Korean J Clin Lab Sci. 2018;50:100-9.
19. Asaad AM, Ansari S, Ajlan SE, Awad SM. Epidemiology of Biofilm Producing Acinetobacter baumannii Nosocomial Isolates from a Tertiary Care Hospital in Egypt: A Cross-Sectional Study. Infect Drug Resist. 2021;14:709-17.

20. Sherif MM, Elkhatib WF, Khalaf WS, Elleboudy NS, Abdelaziz NA. Multidrug Resistant Acinetobacter baumannii Biofilms: Evaluation of Phenotypic-Genotypic Association and Susceptibility to Cinnamic and Gallic Acids. Front Microbiol. 2021;12:716-27.
21. Duarte A, Ferreira S, Almeida S, Domingues FC. Clinical isolates of Acinetobacter baumannii from a Portuguese hospital: PFGE characterization, antibiotic susceptibility and biofilm-forming ability. Comp Immunol Microbiol Infect Dis. 2016;45:29-33.
22. Zhang D, Xia J, Xu Y, Gong M, Zhou Y, Xie L, et al. Biological features of biofilm-forming ability of Acinetobacter baumannii strains derived from 121 elderly patients with hospital-acquired pneumonia. Clin Exp Med. 2016;16:73-80.
23. Simo Tchuinte PL, Rabenandrasana MAN, Kowalewicz C, Andrianoelina VH, Rakotondrasoa A, Andrianirina ZZ, et al. Phenotypic and molecular characterisations of carbapenem-resistant Acinetobacter baumannii strains isolated in Madagascar. Antimicrob Resist Infect Control. 2019;8:31-45.
24. Araújo Lima AV, da Silva SM, do Nascimento Júnior JAA, Correia MDS, Luz AC, Leal-Balbino TC, et al. Occurrence and Diversity of Intra- and Interhospital Drug-Resistant and Biofilm-Forming Acinetobacter baumannii and Pseudomonas aeruginosa. Microb Drug Resist. 2020;26:802-14.
25. Ghasemi E, Ghalavand Z, Goudarzi H, Yeganeh F, Hashemi A, Dabiri H, et al. Phenotypic and genotypic investigation of biofilm formation in clinical and environmental isolates of Acinetobacter baumannii. Arch Clin Infect Dis. 2018;13:120-45.
26. Shenkutie AM, Yao MZ, Siu GK, Wong BKC, Leung PH. Biofilm-Induced Antibiotic Resistance in Clinical Acinetobacter baumannii Isolates. Antibiotics (Basel). 2020;9.
27. Wasfi R, Rasslan F, Hassan SS, Ashour HM, Abd El-Rahman OA. Co-Existence of Carbapenemase-Encoding Genes in Acinetobacter baumannii from Cancer Patients. Infect Dis Ther. 2021;10:291-305.
28. Ghasemi E, Ghalavand Z, Goudarzi H, Yeganeh F, Hashemi A, Dabiri H, et al. Phenotypic and genotypic investigation of biofilm formation in clinical and environmental isolates of Acinetobacter baumannii. Arch Clin Infect Dis. 2018;13:54-90.
29. Zeighami H, Valadkhani F, Shapouri R, Samadi E, Haghi F. Virulence characteristics of multidrug resistant biofilm forming Acinetobacter baumannii isolated from intensive care unit patients. BMC Infect Dis. 2019;19:62-9.
30. Abe K, Nomura N, Suzuki S. Biofilms: hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. FEMS Microbiol Ecol. 2020;96:54-62.
31. Uruen C, Chopo-Escuin G, Tommassen J, Mainar-Jaime RC, Arenas J. Biofilms as Promoters of Bacterial Antibiotic Resistance and Tolerance. Antibiotics (Basel). 2020;10:3.
32. Yang CH, Su PW, Moi SH, Chuang LY. Biofilm Formation in Acinetobacter Baumannii: Genotype-Phenotype Correlation. Molecules. 2019;24:291-305.