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

Abstract

Background: Multiple virulence genes involved in biofilm development by Acinetobacter Baumannii (A. baumannii) like ompA and bap genes. These genes may be analyzed to establish their relevance in biofilm formation or antibiotic resistance phenotypes. This research objects 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 contained 100 isolates including (sputum, endotracheal aspirate, pus from open wounds or open abscesses, mid-stream urine from non-catheterized subjects and catheter-stream urine in catheterized subjects, blood samples and body fluids) were collected from different ICUs. The BD Phoenix Automated System was used to identify the isolates and assess their susceptibility to 21 antibiotics. 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 revealed significant variance 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 found in 44 isolates (44%). The strains having both OmpA and Bap genes (27/27) produce stronger biofilms (100%) than those with just one gene only one gene only ($P <0.001$). The association among the presence of virulence genes and MDR status was measured. The genes encoding OmpA were found at a higher frequency in MDRAB than in non-MDRAB strains ($P=0.014$). Bap gene was found 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 among MDR and the biofilm-forming ability of A. baumannii. Biofilm-related genes (ompA and bap) are connected with multidrug-resistant A. baumannii strains and affect the intensity of biofilm formation.

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

Introduction:

In acute care hospitals, health care-associated infections (HAI) are one of the primary causes of mortality and a significant source of morbidity. A portion of rising morbidity and death is attributable to a rise in antibiotic resistance in HAI, which makes conventional therapy ineffective. It is believed that more than 70 % of HAI-causing bacteria are resistant to at least one routinely used antibiotic [1].

Acinetobacter baumannii (A. baumannii) is a gram-negative bacteria responsible for several illnesses. It is notably prevalent in health care settings, such as hospitals, 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].

A. baumannii strains resistant to a broad-spectrum of antibiotics, such as broad-spectrum B-lactams, aminoglycosides, and fluoroquinolones, have been established and disseminated due to the increased use of antimicrobial chemotherapy in hospitals [3]. A. baumannii is one of the most widespread nosocomial pathogens at now, and is isolated frequently particularly in ICU settings where it causes of serious infections like ventilator-associated pneumonia, secondary meningitis, urinary tract infection (UTI), wound, skin and bloodstream infections. It affects mainly severely debilitated patients, and it is often linked with high morbidity and deaths [4].

Due to the widespread resistance of strains to diverse antimicrobial agents and the ease with which this microorganism spreads and persists in hospital settings, transmission between patients via human reservoirs or inanimate objects, treatment of infections caused by epidemic strains of A. baumannii is frequently challenging [5].

The A. baumannii MDR phenotype have a significant role in the microorganism's exceptional survival and spread in hospital environments, besides its potential to colonize both biotic and abiotic surfaces and to form biofilms [6].

Biofilms are sessile bacterial colonies encased in a matrix consisting of polysaccharide, protein, and DNA. The production of biofilm by bacterial infections is related with increased resistance to host immunological systems, disinfectants, and antimicrobials [7].

It is becoming clear that biofilm synthesis is one of the primary virulence elements shared by a high proportion of A. baumannii clinical isolates [8].

Due to the surface colonization of hospital equipment and indwelling medical devices, such as urinary catheters, central venous catheters, endotracheal tubes, etc., A. baumannii's capacity to form biofilm on abiotic surfaces plays a significant role in the aetiology of nosocomial infections [9].

There are several virulence determinants involved in A. baumannii biofilm development. Analysis of virulence genes may give solid evidence of their relationship with biofilm formation or antibiotic resistance characteristics [10].

The ability of A. baumannii to adhere and create biofilm, its propensity to cause outbreaks and life-threatening infections, and the emergence of antibiotic resistance are all linked [11].

This research objects to study virulence genes associated with biofilm formation of Acinetobacter baumannii to be taken in consideration in prophylactic approaches, treatment, and infection control program.

Materials and Methods:

This research contained 100 samples positive for A. baumannii, collected from subjects 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.

Inclusion criteria were subjects with 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.

Patients were interviewed thoroughly and their medical records were checked (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, DM, 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 biofilm formation detection: This was determined by microtiter plate method using Microtiter plate, 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 obtained using strict aseptic procedures. Sputum samples, Endotracheal aspirates, Bronchoalveolar lavage (BAL), Septic wound swabs, Infected burn swabs, Urine samples and Blood samples were collected.

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 dry sterile with wide neck and 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 was collected from an abscess at the moment it was incised and drained, or when it burst spontaneously. Special care was used while collecting pus from abscesses and wounds to prevent infecting the sample with commensal organisms from the skin. As much as feasible, a wound sample was taken prior to the application of an antiseptic dressing. For infected wounds, a sterile cotton-wool swab was used to acquire a sample. **Urine samples:** In non-catheterized patients: Morning mid-stream urine was kept in sterile, dry, wide-mouthed and leak proof with a tightly fitted lid container after informing the patients to clean the region around the urethral orifice with clean water and soap. In catheterized patients: A sterile syringe was used to collect samples from the distal end of a urinary catheter that had been disinfected with 70 % ethyl alcohol. **Blood samples:** Ten ml of venous blood were withdrawn from each case 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 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. Using the surface streak technique, urine samples were also cultivated on CLED agar by inoculating 1l of well-mixed, non-centrifuged pee using a sterile calibrated bacteriological loop. 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. To calculate the

CFU/ml, the number of colonies collected was multiplied by 1000. 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. Lactose non-fermenting (yellow) colonies characteristic colonies from CLED plates. Following colonial description, pure colonies were gram stained (Secondary gram stain). *A. baumannii* was identified by Phoenix BD with NMIC-431 panels (Becton Dickinson, USA).

AST: All testing were conducted in accordance with the manufacturer's instructions. Phoenix BD with NMIC-431 panels (Becton Dickinson, USA): 25 l of bacteria suspension and a specialized AST indicator were added to Mueller-Hinton broth (MHB). After adding these suspensions to panels placed in the Phoenix BD instrument, the data and their analysis were produced automatically in less than 18 hours 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 cultured overnight at 37°C in TSB with 0.25 percent glucose. The overnight growth was diluted in TSB-0.25% glucose at a ratio of 1:40. Two hundred microliters of cell suspension were injected onto sterile polystyrene microtiter plates containing 96 wells. After 24 hours of incubation, the wells were gently washed three times with 200 microliters of PBS, air-dried, and stained for 15 minutes with 1 % crystal violet. To dissolve crystal violet, the wells were washed with 200 microliters of an ethanol-acetone mixture (80:20 by volume). Microplate reader was used to estimate the optical density at 620 nm (OD620). Each experiment was conducted in triplicate, and the average OD is considered.

For biofilm determination, the following measures were assigned: non-biofilm producer, OD620 0.275; weak biofilm producer, 0.275 OD620 0.55; medium biofilm producer, 0.55 OD620 1.1; and strong biofilm producer, OD620 1.1. The value 0.275 was used as a guideline because it was three SDs above the mean OD (0.303) of a clean microtiter plate dyed using the aforementioned technique.

4- Detection of two virulence genes (ompA & bap) involved in biofilm formation of *A. baumannii*: DNA Extraction Method for Total Genomic Samples According to the GeneJET Genomic DNA Purification Kit Protocol by Thermo Scientific:

1- Pipette 350 L of Lysis Buffer A into a 1.5 mL microcentrifuge tube (not provided). As bacterial pellet, up to 100 mg of fresh or frozen tissue and up to 20 mg of lyophilized tissue were weighed. 100 mg of plant tissue were immersed in liquid nitrogen and finely ground using a mortar and pestle. 100 mg of tissue was inserted in a vial containing beads made of stainless steel. Liquid nitrogen must be used to pre-cool the vial and beads. The configuration of mechanical disruption depends on the kind of tissue.

2- 50 µL of Lysis Buffer B and 20 µL RNase A were added.

3- The sample was incubated for 10 minutes at 65°C with intermittent vortexing or using a shaking water bath, rocking platform, or thermomixer.

4- Add 130 L of Precipitation Solution and thoroughly combine by tilting the tube a few times. On ice, the tube was incubated for 5 minutes.

5- The sample was then centrifuged for 5 minutes at 14,000 rpm and 20,000 g.

6- Typically, The collection and transfer of 450-550 L of supernatant to a clean microcentrifuge tube (not provided). 400 L of Plant gDNA Binding Solution and 400 L of ethanol at 96 % were added and well mixed.

7- 600-700 L of the produced mixture was put to the spin column. It was centrifuged at 6,000 g (8,000 rpm) for one minute. The flow-through solution was discarded, while the remainder of the mixture was applied to the same column. The sample was then centrifuged at 6,000 g (8,000 rpm) for 1 minute.

8- 500 L of Wash Buffer I is added to the column (ensure ethanol has been added to Wash Buffer I). It was centrifuged at 8,000 g (10,000 rpm) for one minute. The flow through was discarded, while the column was reinserted into the collecting tube.

9- 500 L of Wash Buffer II should be added to the column (ensure ethanol has been added to Wash Buffer II). It was centrifuged for 3 minutes at 20,000 g (14,000 rpm) maximum speed. The tube of collecting was empty. The purification column was reinserted into the tube and spun at full speed (20,000 g, 14,000 rpm) for one minute. The flow-through solution-containing collecting tube was withdrawn, and the column was transferred to a 1.5 mL sterile microcentrifuge tube (not provided).

10- To elute genomic DNA, 100 L of Elution Buffer was added to the centre of the column membrane, it was left at room temperature for 5 minutes, and it was centrifuged for 1 minute at 8,000 g (10,000 rpm).

11- 100 L Elution Buffer was used for the second elution stage. The second elution might be performed in the same elution tube or in a different tube. The DNA was suitable for use in subsequent applications or storage at -20°C.

PCR amplification:

DreamTaq Green PCR Master Mix (2X) (K1081, Thermo Fisher, USA) was used to amplify specified genes using a Creacon (Holland, Inc) PCR system cycler in accordance with the manufacturer's instructions. 22 cycles consisting of initial denaturation at 95 ℃ for 60 seconds, denaturation at 94 ℃, annealing at 50 ℃, extension at 72 ℃ for 60 seconds, and final extension at 72 ℃ for 3 minutes [13].

Agarose gel electrophoresis and detection of the amplification products:

In a 50ml flask, 0.75g of agarose was mixed with 50ml of 1x TBE electrophoresis buffer to produce a 1.0 % agarose solution. The agarose was then dissolved using microwave heating. At 50°C, the agarose was cooled. A comb was placed on the electrophoresis bed, and agarose was then poured in.

When pouring agarose, care must be taken to avoid the formation of bubbles. The gel solidified within 15 minutes and turned cloudy, the electrophoresis apparatus was filled with electrophoresis buffer, and the comb was removed to create 6 or 10 wells for sample application) in the presence of a DNA ladder (peqGOLD 1 kb DNA-Ladder, Peqlab, VWR) in accordance with the manufacturer's instructions. Electrodes were linked to the power source, which was then activated. It was set for 100 minutes at 80 Volts. Gel was removed from its bed and put on the gel staining tray for 30 minutes of staining with Ethidium bromide and 20 minutes of destaining in distilled water.

Data analysis:

Gel documentation system (Geldoc-it, UVP, England) was used with total lab analysis software for data processing (ww.totallab.com, Ver.1.0.1). The elution of positive amplicons of 1500 bp from an agarose gel. PCR products were purified using Micro spin filters and quantified using spectrophotometry. Using the ABI PRISM® 3100 Genetic Analyzer, sequence analysis was achieved (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 entered into the computer and analyzed using version 20.0 of the IBM SPSS software suite. (Armonk, New York: IBM Corporation) Quantitative and percentage descriptions were provided for qualitative data. The Kolmogorov test was employed to establish the distribution's normality. The range (minimum and maximum), mean, standard deviation, median, and interquartile range (IQR) were used to characterise quantitative data. The acquired findings were deemed significant at the 5 percent level. For categorical variables, the Chi-square test was employed to compare various groups. Fisher's Exact statistic for chi-square correction when over 20% of the cells have an anticipated count of less than 5. Kruskal Wallis test for quantitative variables with an anomalous distribution when comparing more than two groups, and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons. A p value less than or equal to 0.05 was deemed 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 techniques, while the majority were biofilm techniques (71%). The incidince of weak biofilm techniques was (17%), (27%) moderate biofilm techniques, and (27%) strong biofilm techniques. The median OD620 and IQR measures for non-biofilm techniques was 0.21 (0.11, 0.26), for weak biofilm techniques, 0.39 (0.29, 0.51), moderate biofilm techniques, 0.79 (0.59, 0.99) and strong biofilm techniques, 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 biofilm biomass OD_{620} comparison among various sources of specimens showed significant variance 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

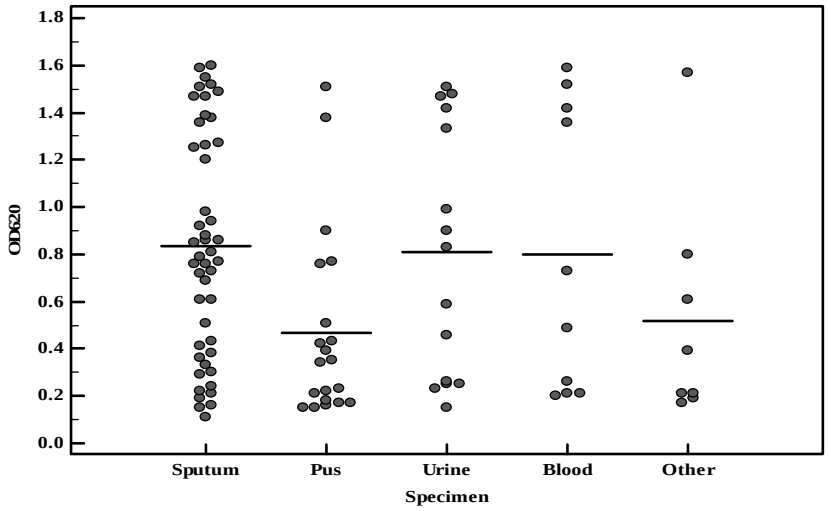

Figure 1: Relation between specimen and OD_{620}

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

12 antibiotics were evaluated for their effectiveness against every isolate. Most isolates exhibited gentamicin resistance (70%). The isolated isolates of A. baumannii were likewise 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 A. baumannii isolates were classified as multidrug-resistant (MDRAB) when they exhibited resistance to three or more antibiotic classes. The prevalence 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 variance 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 among biofilm forming capacity and individual drug resistance of *A. baumannii* was measured. The resistance rates of most antibiotics were shown to be higher in biofilm-forming than non-biofilm forming groups with a P-value ranging from 0.001 to 0.5. 56 (78.9%) of the 70 gentamicin-resistant isolates were biofilm makers, whereas only 6 of 21 (8.5%) of the gentamicin-susceptible isolates were biofilm technique (P <0.001; Monte Carlo test). In *A. baumannii* that resistance to gentamicin had an increased ability to build biofilms in comparison 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 association was detected statistically. **Table 7**

Table 7: Relation between Biofilm and Antibiotics

Antibiotics		Biofilm				$\chi 2$	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 prevalence 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 found in 44 isolates (44%). The strains having both OmpA and Bap genes (27/27) form stronger biofilms (100%) than those with just 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**

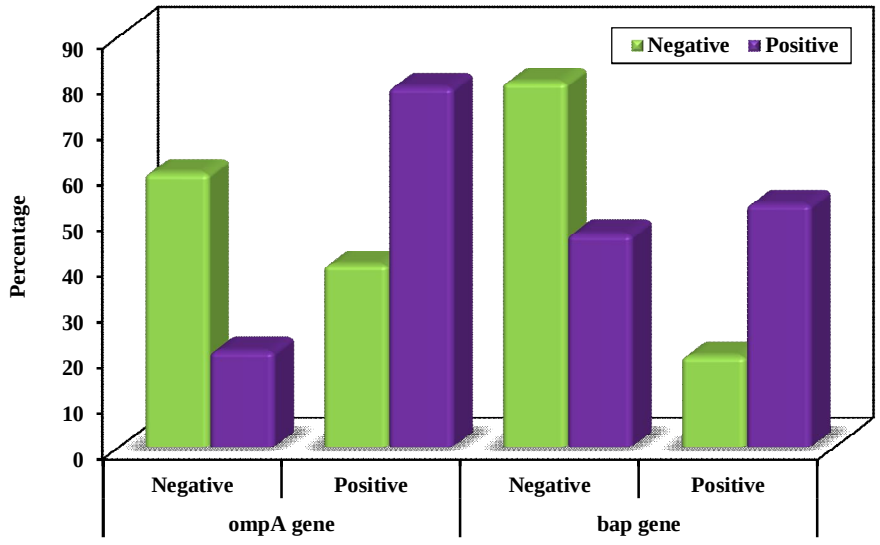

Figure 2: Relation between MDR with OmpA gene and Bap gene.

Discussion

Acinetobacter baumannii is regarded as a hazard 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 current work, we determined the biofilm formation in the clinical isolates. It was found that 71% of examined A. baumannii demonstrated biofilm forming capacity. This was consistent 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 produced biofilms respectively.

Several studies have connected a high frequency of biofilm formation in A. baumannii with increased survival and resilience to environmental challenges, such as nutrition limitation and dehydration [18]

In Egypt, Asaad et al. [19] revealed that 70.1% of A. baumannii clinical isolates showed biofilm formation, while Sherif et al.[20] , found that A. baumannii isolates had a much greater frequency of 100% biofilm production.

The assessment of biofilm biomass (OD620) from different specimen sources. Our findings 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 outcomes were also in line with the outcomes informed 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 cling and form biofilms specially in flowing settings, which lead to chronic infections.

Regarding the individual analysis of biofilm forming ability of different isolates. our findings agreed with Zhang et al. [22], who observed 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 RTIs including health care associated pneumonia.

The high prevalence rates of Multidrug resistant Acinetobacter baumannii (MDRAB) clinical isolates have been recorded globally, ranged from 21–95%. [22] Similar to prior publications, we noticed a significant incidence of MDRAB in our sample; 90% (90/100) of our isolates exhibited resistance patterns similar with other reports. [23, 24]

In the current study, we explored the association among multidrug resistant Acinetobacter baumannii (MDRAB) and biofilm strength, it was found that there was a significant correlation ($p < 0.05$) among biofilm strength and MDR, which agreed with Ghasemi et al.[25], who found that all strong biofilm producing A. baumannii isolates were MDR, and hence there was a significant correlation among strong biofilm synthesis 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], indicated an inverse relationship among biofilm synthesis of A. baumannii and antibiotic resistance. They found that the capacity of bacteria to build biofilm may be connected with antibiotic resistance at the individual point. There was a statistically significant correlation among antibiotic resistance and certain antibiotics.

In the present study, 78.9% of biofilm producing strains were resistant to gentamycin with p value < 0.05. This outcome was like the research of Thummeepak et al.[15], who observed that Biofilm-forming A. baumannii strains were more likely to be resistant to gentamicin. The co-location of biofilm-associated and gentamicin resistance determinants on the same plasmid or genomic island may account for this phenotypic relationship. Also, Duarte et al.[21], found that Biofilm formation was more prevalent among gentamicin- and tobramycin-resistant A. baumannii strains than among 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 findings agreed with the findings 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]

A. baumannii biofilm-related genes, notably the OmpA and Bap genes, have been implicated in biofilm formation and antibiotic resistance in many studies. [28] The purpose of this investigation was to confirm the presence of virulence genes (OmpA and Bap) in A. baumannii isolates and their relationship to 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 researches 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 outcomes showed that 50% of A. baumannii isolates possessed Bap gene. This was in agreement with Thummeepak et al.[15], who described that 48% and 30% of A. baumannii isolates were positive for Bap gene respectively.

In the current research, when analyzing the correlation among biofilm development and the related virulence genes, a significant positive correlation was found among biofilm formation and the existence of 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.

Due to the potential of horizontal gene transfer between isolates in close proximity inside biofilms, this may be of grave concern.[30, 31]

These findings were correlated with the those of Liou et al. and Fallah et al., who have indicated that biofilm-associated genes, such as OmpA and Bap, were attributed to the biofilm formation of A. baumannii strains.

On the other hand, Thummeepak et al. [10] discovered that A. baumannii isolates containing virulence genes didn't increase biofilm development on polystyrene, but the incidence of Bap or OmpA correlated negatively with biofilm formation.

Remarkably, the present study detected a statistically significant association among 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 deemed to have a high diagnostic value for distinguishing the biofilm-forming phenotype of A. baumannii.

In the present study, we presented a significant correlation among MDRAB strains and the incidince of OmpA or Bap genes. In agreement with these results, Thummeepak et al.[10], found a strong association among MDR A. baumannii phenotype and the existence of Bap and OmpA genes. Due to the fact that OmpA is a -barrel porin, it is feasible that antibiotics

are transmitted from the periplasm via the outer membrane and subsequently link with efflux pumps in the inner membrane.

Therefore, the transcriptional or translational investigation of virulence genes of A. baumannii might give useful information to establish their link with biofilm or antibiotic resistance phenotypes, which must be examined in further detail.

Conclusions:

Acinetobacter baumannii is a major source of hospital-acquired illnesses and an opportunistic bacterial disease. 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) are connected with multidrug-resistant A. baumannii strains and affect the intensity of biofilm formation.

Ethical Approval and consent:

The research was performed after being approved from the institutional ethical committee, Tanta University. All subjects included in the study provided an informed written consent.

Financial support and sponsorship: Nil

Conflict of Interest: Nil

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.