ORIGINAL ARTICLE

Multi-drug Resistance Profile of Virulent Biofilm forming *Acinetobacter baumanii* isolates in Intensive Care Units

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ABSTRACT

Background: *Nosocomial infection with multidrug resistant biofilm forming Acinetobacter baumanii is a serious problem in healthcare settings with worldwide concern.* ***Objectives:*** *To study the prevalence and antimicrobial resistance profiles of Acinetobacter baumanii isolates detect biofilm formation and correlate biofilm FimH and bap genes with drug resistance.* ***Methodology:*** *This was a retrospective study performed in medical microbiology and immunology department, faculty of medicine, Menoufia university hospitals. Acinetobacter baumannii were isolated from intensive care units and phenotypically tested for biofilm formation by modified congo-red method. Antimicrobial susceptibility was evaluated by disc diffusion methods according to CLSI guidelines. Virulence genes (FimH and bap) concerned with biofilm formation were screened using polymerase chain reactions.* ***Results****: A total of 50 Acinetobacter isolates in ICUs were collected from 215 different clinical samples (23.3%). MDR was detected in 20 isolates and more than 28 isolates had formed biofilms. Both fimH & bap genes were detected by 58% and 30% combination rate with a significant correlation with virulence and drug resistance profiles.* ***Conclusion:*** *MDR Acinetobacter in ICUs has greater pathogenicity potentials emphasizing the importance of early detection using available laboratory techniques.*

INTRODUCTION

Over the past 20 years, antimicrobial resistance (AMR) of Gram-negative bacteria has become a dramatic global problem for healthcare systems, reflecting limited treatment options with high costs and death rates. Gram-negative species are frequently associated with nosocomial infections, including bloodstream infections, hospital acquired pneumonia, urinary tract infections, skin and soft-tissue infections [1]

The World Health Organization (WHO) had published the priority list of antibiotic-resistant bacteria, identifying the most significant species globally. This list includes multi drug-resistant *Acinetobacter baumannii* (MDRAB) as "critical" pathogen with premium priority among critically ill patients. [2]

To survive antibiotics, bacteria use different strategies: either by expression of resistance genes or evading their effects e.g. by biofilm formation and/or persisting inside host cells. *Acinetobacter baumannii* have gained resistance through a variety of β-lactamase enzymes: initially through penicillinases, then cephalosporinases, extended-spectrum βlactamases (ESBLs) and carbapenemases [3]

The biofilm-associated protein (Bap) is a surface-exposed, highly divergent protein that is playing important role in intercellular adhesion, accumulation of bacterial cells, and biofilm establishment. Also adhesin fimbriae promote antibiotic evasion through increasing bacterial adhesion and internalization by macrophages. *FimH* adhesion is the most common adhesive organelle in different enterobacteria particularly in biofilm producing strains. [4-5]

An evidence-based knowledge regarding virulence and antimicrobial resistance pattern is fundamental for guiding empirical therapy of critical pathogens. This guide is also important for effective antimicrobial stewardship as well as in the design of local and universal research programs [6]

METHODOLOGY

This study was done in Medical Microbiology and Immunology Department, Faculty of medicine, Menoufia University during the period from August 2021 to August 2022. A full patient history was taken. Personal history included: name, age, sex, antimicrobial administration. Clinical history included: date and cause of ICU admission, exposure to invasive procedures as central venous lines and endotracheal tubes and associated co-morbidities.

This study protocol was approved by the local Ethics Committee of the Menoufia University (9/2022 SURG6). Full consents were obtained from all patients.

Processing of the samples:

Different clinical samples (sputum, blood, urine, wound swabs) according to the site of infection were collected from hospitalized patients. These collected specimens were immediately delivered to microbiology laboratory to be processed and examined. Each sample was inoculated on blood agar, MacConkey's agar, mannitol salt agar, and CLED agar plates (for counting of microorganisms in urine) and incubated aerobically at 37°C for 24-48h. All inoculated blood culture bottles were incubated up to 7 days and subcultured every 48 hours on blood agar and Mac Conkey medium [7]

Identification of Acinetobacter:

Each non lactose fermenting colony on MacConkey agar media was picked up. *Acinetobacter* species were isolated and identified as Gram negative, catalase positive, oxidase and urease negative, sugar non fermenter confirmed by Vitek compact system (bioMerieux, France).

Antimicrobial susceptibility testing:

Susceptibility screening tests were done for all Acinetobacter isolates by disk diffusion method and interpreted according to recent Clinical and Laboratory Standards Institute guidelines using different antibiotic susceptibility disks (Oxoid): piperacillin (100 µg), cefepime (30 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10µg), tetracycline (30 µg), doxycycline (30µg), ciprofloxacin 5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg).Screening detection of ESβL production was done using Ceftazidime, Cefotaxime & Ceftriaxone (30 µg) and confirmed by Cephalosporins/ Clavulanate combination test. Also screening the resistance to carbapenems was detected using imipenem disk diffusion method. Results were categorized as resistant if zone diameter was ≤ 18 mm [8]

Phenotypic detection of biofilm formation:

Modified Congo red agar method (MCRA):

Congo red agar (CRA) is comprised of brain heart infusion broth 37 g/L, sucrose 50 g/L, Congo red dye 0.8 g/L, agar 10 g/L and 1000 ml water. The media in modified Congo red agar consists of Congo red dye 0.4 g/L, Blood Base Agar -2 40 g/L, glucose 10 g/L and 1000 ml water. The stain was prepared as concentrated aqueous solution and autoclaved (121°C for 15 min) separately from other medium constituents and was then added when the agar has cooled to 55°C. Inoculated agar was incubated for 48 h at 37°C and subsequently 2-4 days at room temperature. Black color interpreted as positive biofilm producing strains with variable strength in contrast with red colonies which was interpreted as negative biofilm producers **(Ahmed B. Mahmoud et al.)** [9]

Molecular detection of biofilm formation fimH & bap genes:

DNA extraction was carried out using the Thermo Scientific gene JET™ genomic DNA Purification Kit and a Microcentrifuge applying manufacturer instructions. The concentrations of DNA were assessed using the NanoDrop™ 2000 system (Thermo Scientific, USA). The DNA extracts were kept at -80ºC.

PCR reactions were performed in a 25µl total volume using PCR thermocycler (Biometra, Germany) as follows: we added 12.5 µl DreamTaq green PCR Master Mix (2x), 1 µl *FimH* forward primer F: 5`TGCAGAACGGATAAGCCGTGG 3`, 1 µl *FimH* reverse primer R: 5`GCAGTCACCTGCCCTCCGGTA 3`[10], 10 µl DNA Extract plus 0.5 µl water nuclease –free. The thermo-cycling conditions were modified as follows: Initial denaturation at 95°C for 4 min followed by denaturation at 94°C for 1 min, annealing at 60°C for 45sec, extension at72°C for1 min with final extension at72°C for 10 min. Similar steps were done for *bap* gene, forward primer F:5` TGCTGACAGTGACGTAGAACCACA 3` & reverse primer R: 5` TGCAACTAGTGGAATAGCAGCCCA 3`[5]. Agarose gel electro-phoresis was carried out and DNA bands were visualized (*FimH* & *bap* give a band at 508 & 184 bp, respectively).

Statistical analysis:

The data collected were tabulated & analyzed by SPSS (statistical package for the social science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows. Two types of statistics were done; Descriptive statistics: were expressed as number and percentage %.

RESULTS

Two hundreds and fifteen clinical samples were collected from hospitalized adult patients (one sample from each patient). All the selected patients had infections that became evident 48 hours or more after hospital admission. A total of 50 Acinetobacter isolates were obtained from 215 different clinical samples. Acinetobacter infections were more common in males (64 %) aged from 40-70 years (68%), who stayed in ICUs for more than 10 days (88%), antimicrobial administrated (94%), exposed to invasive procedures (86%) and had associated co-morbidities (90%).The highest rate of Acinetobacter isolates was from respiratory samples (56%). Vitek system showed that *A. baumannii* was the most isolated *Acinetobacter* spp. (80%).

Antimicrobial susceptibility testing by screening disk diffusion method detected high resistance to cefepime, tobramycin (88% for each), ceftriaxone and pipercillin (86% for each), tetracycline (76%) also resistance to gentamycin, amikacin, ceftazidime, ciprofloxacin by more than 50%. Twenty-three isolates were detected as carbapenems resistant by imipenem disk diffusion method. Twenty isolates were multidrug-

resistant according to centers for disease control (CDC) guidelines.

About 60 % of the isolated Acinetobacter species were ESβLs-producers compared to 32% for combined disk confirmatory test. About (56%) of Acinetobacter isolates were biofilm producers by modified congo-red agar method in line with (58%) for genotypic detection (**figure 1 & 2**)

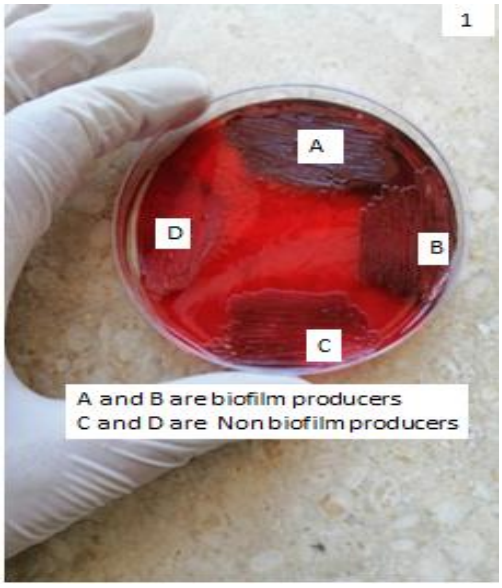

Fig. 1: Biofilm detection by modified congo-red method

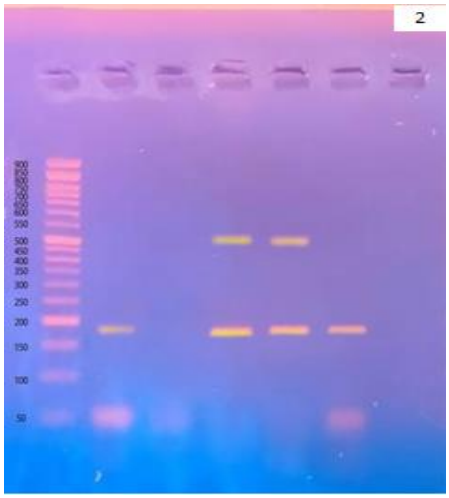

Fig. 2: Ultra violet trans-illumination detected DNA bands at 508 & 184 bp for FimH & bap genes respectively among acinetobacter isolates.

There was significant association ($p<0.05$) between the biofilm formation and antibiotic resistance in general as shown in **figure (3)**. Antimicrobial susceptibility testing of ESβL-producing Acinetobacter isolates showed marked resistant to piperacillin, ceftazidime, cefepime, ceftriaxone (100% for each), cefotaxime (93.8%),gentamycin, ciprofloxacin (81.25% for each), amikacin (75%), imipenem (62.5%). About 44% of ESBL producers were from respiratory secretions.

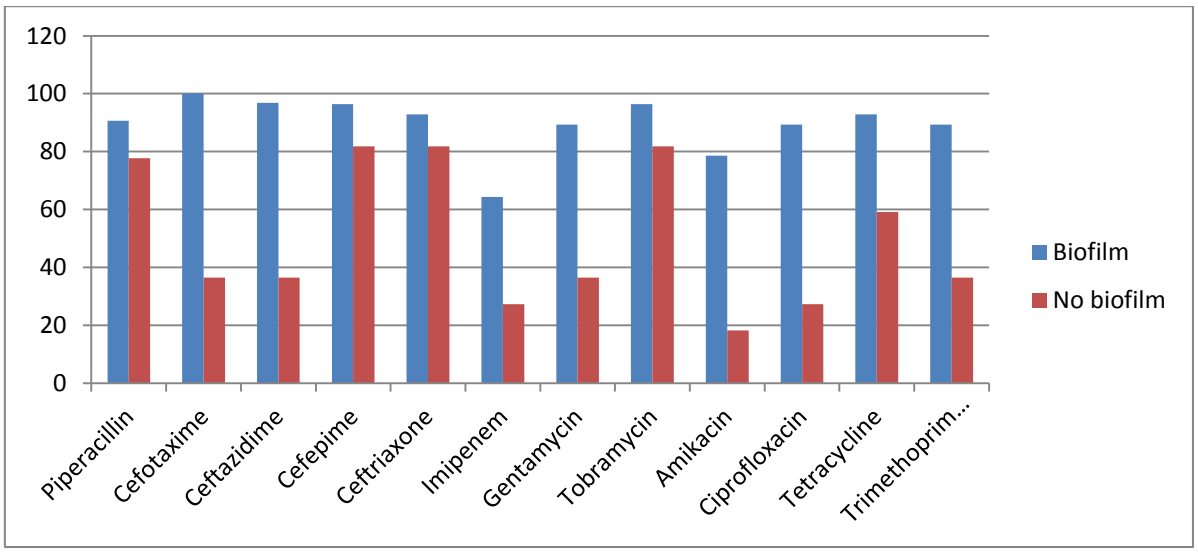

Figure (3) Biofilm formation in relation to antibiotic resistance among isolates

There was significant association ($p<0.05$) between the biofilm formation and for ESβL-production as shown in **Table (1).** Also a statistically significant association was detected between biofilm formation and carbapenems resistance as shown in **Table (2)**

Table 1: Biofilm formation & ESβL-production among isolates

ESBL	Biofilm formation				Total (n= 50)	
	Positive (n=29)		Negative (n=21)			
	No.	%	No.	%	No.	%
Positive	13	81.25	3	18.75	16	**32**
Negative	16	47.05	18	52.95	34	**68**
Total	**29**	**58**	**21**	**42**	**50**	**100**

Table 2: Biofilm formation & carbapenem resistance among isolates

Carbapenem resistance	Biofilm formation				Total (n= 50)	
	Positive (n=29)		Negative (n=21)			
	No.	%	No.	%	No.	%
Positive	18	78.3	5	21.7	23	46
Negative	11	37.9	16	62.1	27	54
Total	29	58	21	42	50	100

Both *fimH* & *bap* genes were detected among *Acinetobacter* isolates by 58% as shown in table 3 and 4**.** Both genes were detected by 30 % among isolates**.**

Table 3: Detection of *fimH* & Biofilm formation among isolates

fimH gene	Biofilm formation				Total (n= 50)	
	Positive (n=29)		Negative (n=21)			
	No.	%	No.	%	No.	%
Positive	24	82.8	5	17.2	29	58
Negative	5	23.8	16	76.2	21	42
Total	29	58	21	42	50	100

Table 4: Detection of *bap* & Biofilm formation among isolates

bap gene	Biofilm formation				Total (n= 50)	
	Positive (n=29)		Negative (n=21)			
	No.	%	No.	%	No.	%
Positive	26	89.7	3	10.3	29	58
Negative	3	14.3	18	85.7	21	42
Total	29	58	21	42	50	100

FimH positive isolates showed high resistance to tobramycin, cefepime, ceftazidime and tetracycline by 93.1%, 89.6%, 86.2% and 82.7% respectively. About 62% of *fimH* positive species (18/29) showed multi drug resistance pattern. Similarly *bap* positive isolates presented multi-drug resistant rate exceeding 65% especially for ceftazidime, tetracycline, imepenem & amikacin by 86.2%, 89.6%, 68% and 69% respectively. Significant correlation was detected between the biofilm formation and both *fimH & bap* PCR detection in Acinetobacter isolates. About 26% of Acinetobacter isolates were *fimH* & *bap* positive, biofilm forming plus multidrug resistant strains.

DISCUSSION

Acinetobacter spp. especially *A. baumannii* has received much attention in recent years because of its increasing involvement in severe infections and outbreaks occurring in clinical settings, and presumably related to its ability to survive and persist in hospital environments [11]

In the current study, hospital acquired *Acinetobacter* infections were detected by more than 23%. Most patients were hospitalized for more than 10 days (88%), used chemotherapeutic agents (94%), exposed to invasive procedures (86%) and had associated co-

morbidities (90%). Such findings came in accordance with Kanafani et al., [12]

In this study, the higher isolation rates (56%) of *Acinetobacter* spp. from the respiratory samples are in agreement with Fam et al., [13]. This could be explained by increased mechanical ventilation rates with COVID-19 patients resulted in ventilator-associated pneumonia (VAP), especially with multidrug-resistant bacteria such as *A. baumannii*. However, Zowawi et al., [14] found that *Acinetobacter* isolates was mainly isolated from wound swabs (39%) followed by sputum (22%) and urine samples (6%). Also, Kaur et al.[15] in India reported that majority were from urine (26.1%), vaginal swabs (23.2%), pus (18.3%), blood (17.6%) followed by other samples. They found that *A. baumannii* complex was the predominant isolated species by 91.6% in line with our results (80%).

Upon applying disk diffusion method for assessment of antibiotic susceptibility pattern, *Acinetobacter* spp. isolates displayed higher levels of antibiotic resistance for most of the used antibiotics. The antimicrobial susceptibility profile also revealed that 20 (40%) *Acinetobacter* spp. isolates were MDR. These results agreed with that reported by previous studies [13]. Some researchs [15-16] revealed higher rates of MDR among the isolated *Acinetobacter* spp. (73.24%).

About 32% of *Acinetobacter* spp. isolates in this study were ESβL producers which came in concordance with Abd El-Baky et al.[17] Lower rates were reported by Ranjbar et al.[18] who reported that ESBL production rate of *Acinetobacter* spp. isolates was less than 19%.

Extent of resistance exhibited by *Acinetobacter* spp. against different classes of antibiotics results in failure of the various treatment strategies in hospital set-ups. The elevated rates of multidrug resistance *Acinetobacter* spp. are further aggravated by its ability to form biofilms [19]. Antibiotic resistance in biofilms is estimated to be ~1,000-fold higher than in planktonic cells, thus restricting available choices of effective antimicrobial treatments [20]

The congo-red detection method was an accurate method by about 97% in comparison to genotypic method with 58% of *Acinetobacter* spp. isolates biofilm producers matching results of Shenkutie et al.[21] who have demonstrated that 53.97% and 59.6%, respectively were biofilm producers in contrast to Ghasemi et al.[22] who reported that only 7.1% of the bacteria were biofilm producer.

The current study highlighted a significant association between biofilm formation and antibiotic resistance as there was a highly significant statistical difference between biofilm-producers and non-producers. Similar results were obtained by Yang et al., [16] On the other hand, a study done by Shenkutie et al.[21] have documented an inverse relation between antibiotic resistance and biofilm-forming ability of the isolates.

Moreover, this study showed that 81.25% of ESβL-producing *Acinetobacter* spp. isolates were biofilm-positive. However, Abd El-Baky et al., [17] reported lower rates of ESβL-producing Acinetobacter spp. isolates (35%).

Generally, carbapenems are found to be the most active antimicrobials against *Acinetobacter* spp. [12] However, 42 % of this study isolates were found to be resistant to imipenem antibiotic. This is in line with recent reports on increasing carbapenem resistance among bacteria[23]**.** Other studies[24-25] reported higher rates of carbapenem resistance among *Acinetobacter* isolates; 88.9% and 100%, respectively.

In the present study, the PCR assay revealed that 58% of the *Acinetobacter* spp. isolates harbored *fimH* gene. Similar results were reported in study conducted by Ahmed et al.[26] while Zeigham et al.[27] reported that the *fimH* gene was not detected at all in any *Acinetobacter* isolates.

When analyzing the correlation between biofilm formation and the related *fimH* gene in the present study, we found that among *Acinetobacter* isolates, 24/29 (82.8%) of the biofilm-producing *Acinetobacter* isolates were positive for the *fimH* gene. Similar results were reported in studies conducted by Altınok et al. [28] with high antimicrobial resistance pattern.

In our study, we reported a significant correlation between the biofilm formation and *bap* gene required for adherence to bronchial cells and structural integrity and water channel formation within the biofilm. Our results were similar to previous researches[29-30]

Furthermore, the *fimH and bap* genes carriage rate of *Acinetobacter* isolates in MDR was significantly higher than that of non-MDR isolates in accordance with Asaad et al.[31]**.** This suggests that the drug resistant *Acinetobacter* isolates seem to have greater pathogenicity potentials.

The present study revealed that the occurrence rates of biofilm-producing genes are nearly similar to the results observed in the MCRA method. Similar findings were reported by previous studies[32]. However, different rates observed in the different phenotypic and molecular assays were recorded others [33]. Different factors the expression among different adhesion genes regulate biofilm formation, which can be triggered by various environmental factors such as the concentration of glucose in the used media, the temperature of the contact surfaces, the osmolality of the used media, and the growth conditions of the organisms plus a discrepancy in the regulation of locus genes' and putative adhesion genes' expression or the emergence of insertional inactivation and point mutations in the locus of adhesion genes.

CONCLUSION

The high rates of multi-drug resistance in ICUs are ghosts for doctors emphasize the great importance of infection control strategies to decrease infection rates. Most MDR *Acinetobacter* isolates were virulent biofilm forming strains making application of monitored disinfection programs side by side with effective antimicrobial stewardship policies our priority.

The variations of biofilm detection methods may lead to different observations among different researchers. MCRA method is an accurate, cheap, easy applicable biofilm detection method. However, an integration of both phenotypic and genotypic tests could be used to identify biofilm-producing bacterial isolates fully accurate.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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