ORIGINAL ARTICLE

Biofilm Formation by *Acinetobacter* Species Isolated from Intensive Care Units: Unveiling the Impact on Antibiotic Resistance

[1]Shaimaa M. Seleim, [1]Mona A. Abd El-Massih, [2]Mohamed H. Saleh, [1]Doaa M. El-Wakil*
[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Cairo, Egypt
[2]Department of Critical Care, Faculty of Medicine, Cairo University, Cairo, Egypt

Key words:
Acinetobacter species, biofilm formation, ICUs, MDR, XDR

Corresponding Author:
Doaa Mahdy El-Wakil, M.D.
Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Al-Saray Street, Al-Manial, Cairo, 11559, Egypt
Tel: +201222320826
Fax: +201222320826
do3aamahdy@cu.edu.eg
ORCID: 0000-0002-3863-5664

ABSTRACT

Background: *The potential ability of Acinetobacter species to form biofilm could explain their remarkable antibiotic resistance in intensive care settings.* ***Objectives:*** *This work aimed to detect the biofilm formation by Acinetobacter species and investigate its impact on their antibiotic resistance pattern.* ***Methodology:*** *A total of 50 non-replicate Acinetobacter isolates, recovered from patients admitted to intensive care units (ICUs), were collected from the Clinical Laboratories of Kasr Al-Ainy University Hospitals. The isolates were subjected to antibiotic susceptibility testing against nine antibiotics of different classes using the Kirby-Bauer disk diffusion method, while the broth microdilution (BMD) method was used to determine polymyxin B susceptibility. The ability to produce biofilm was determined via the tissue culture plate (TCP) method.* ***Results:*** *Forty-nine (98%) isolates were biofilm formers, of which 53.1% were moderate biofilm formers that predominated over the strong and weak biofilm formers (38.8% and 8.1%, respectively). All cases with CAUTIs were infected with moderate biofilm formers (100%, p = 0.045), while the strong biofilm formers displayed a statistically significant higher resistance rate (68.4%) to cotrimoxazole (p = 0.048). Although the strong biofilm formers among multidrug-resistant (MDR) isolates were higher than that of extensively drug-resistant (XDR) isolates (62.5% and 34.1% respectively), the difference was statistically insignificant (p = 0.442).* ***Conclusions:*** *The high rate of biofilm-forming Acinetobacter isolates could potentially increase the colonization by MDR and XDR bacteria in intensive care settings. Isolates with a lower level of resistance exhibited more robust biofilm, which necessitates the urgent finding of effective preventive measures against biofilm formation.*

INTRODUCTION

Acinetobacter species are Gram-negative, saprophytic, non-fermentative coccobacilli that are recognized as significant nosocomial pathogens, because of their ability to sustain a broad range of dry and wet surfaces in healthcare settings[1]. Among the *Acinetobacter* spp., *A. baumannii* is known as the most frequently isolated organism in intensive care units (ICUs)[2], causing a variety of nosocomial infections, including pneumonia, urinary tract infections (UTIs) as well as skin and soft tissue infections[3]. These infections are usually associated with high mortality rates ranging between 26% among hospitalized patients and 43% among ICU patients[4].

The terms multidrug resistance (MDR), extensive drug resistance (XDR), and pandrug resistance (PDR) have all been used to describe the level of antimicrobial resistance in *Acinetobacter* spp., however, there is no generally recognized definition for bacterial resistance[5]. The Centers for Disease Control and Prevention (CDC) classifies carbapenem-resistant *Acinetobacter* as an urgent threat that necessitates immediate management, continual public health monitoring, and prevention strategies[6]. Moreover, it has been reported that several *A. baumannii* nosocomial isolates showed resistance to colistin and tigecycline, the last resort antibiotics prescribed in the treatment guidelines[7].

Biofilm is a microbial community that forms on hard surfaces and is frequently embedded in a dense matrix of extracellular polymeric substances (EPS) that renders them resistant to antibiotics, thus very difficult to manage[8]. It has been estimated that approximately 98% of *Acinetobacter* infections are caused by biofilm-forming strains[9]. The ability of *Acinetobacter* spp. to form biofilm that enables bacterial survival in hospital settings, especially in ICUs, is the most significant contributing factor to their virulence, and this trait is also responsible for their notable antibiotic resistance[10].

Several biofilm-related genes influence antimicrobial susceptibility, suggesting an association between the biofilm-forming ability of *Acinetobacter* spp. and their antibiotic resistance patterns (MDR/XDR)[11,12]. However, previous studies have reported contradictory results concerning this relationship[13,14]. Thus, it could be helpful to enhance

infection control measures in healthcare settings by providing new insight into the potential link between biofilm formation and antibiotic resistance patterns of *Acinetobacter* nosocomial isolates[7].

The aim of this study was to determine the ability of *Acinetobacter* spp. to produce biofilm and to detect their antibiotic resistance pattern (MDR/XDR and non-MDR/XDR). It also attempted to explore the possible association between the strength of biofilm formation and antibiotic resistance patterns.

METHODOLOGY

Study design and setting:

This cross-sectional analytic study was conducted over 6 months, from March 2018 through August 2018. A total of non-replicate 50 nosocomial isolates of *Acinetobacter* spp. were collected from the Clinical Laboratories of Kasr Al-Ainy University Hospitals and were retrieved from patients admitted to ICUs. The isolates were recovered from different clinical samples, including 12 endotracheal aspirates (ETA), 15 sputum, 12 urine, and 11 wound swabs. Patients' demographics, clinical data, and outcomes were gathered retrospectively from medical records. During the hospital stay, the patients were managed according to the fourth edition of Surviving Sepsis Campaign Guidelines 2016[15], which were the latest at the time of the study. All isolates were transferred to the Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University for further testing. The study protocol has received the approval of the Faculty of Medicine, Cairo University, Egypt on 25/2/2018. This work has been conducted in accordance with the principles outlined in the Declaration of Helsinki.

Culture and identification of *Acinetobacter* isolates:

Each of the obtained isolates was subcultured on MacConkey's medium (Oxoid, Hampshire, UK) and incubated aerobically at 37 °C for 24 hours. Identification of *Acinetobacter* spp. was confirmed by the conventional microbiological methods[16], including colony morphology, motility test, Gram-stained smear, and biochemical reactions, including oxidase, catalase tests, and triple sugar iron (TSI). *Acinetobacter* isolates were identified as colorless or light lavender colonies, non-motile, Gram-negative, oxidase-negative, catalase-negative, and non-fermentative coccobacilli.

Antibiotic susceptibility testing:

The Kirby-Bauer disk diffusion method was used to evaluate the *in vitro* susceptibility of *Acinetobacter* clinical isolates against the following antibiotic agents (Himedia, India): piperacillin (100 µg), ampicillin/sulbactam (10+10 µg), ceftriaxone (30 µg), cefepime (30 µg), imipenem (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), tetracycline (30 µg), and trimethoprim-sulfamethoxazole (TMP-SMX) (1.25+23.75µg). The inhibition zone diameter was measured in mm and interpreted as susceptible, intermediate, or resistant using the CLSI breakpoints[17]. *Pseudomonas aeruginosa* ATCC 27853 (The Central Laboratories, Cairo, Egypt) was used as a quality control strain for susceptibility testing. Determination of polymyxin B (Himedia, India) susceptibility was done using the broth microdilution (BMD) method according to the protocol suggested by the CLSI[17]. The polymyxin B concentrations used for testing ranged from 64 µg/ml to 0.25 µg/ml. Controls for each plate were prepared using sterile cation-adjusted Mueller-Hinton (CAMH) broth (Oxoid, Hampshire, UK) as sterility control and the bacterial suspension as growth control. Results were interpreted according to the following breakpoints recommended by the CLSI guidelines for polymyxin B: ≤2 µg/ml is considered susceptible, while ≥ 4 µg/ml is considered resistant[17]. According to the definitions stated by *Magiorakos et al.*[5], the terms "MDR" and "XDR" were used to describe the resistance pattern of *Acinetobacter* spp.

Biofilm formation testing by tissue culture plate (TCP) method:

To determine the biofilm-forming ability of *Acinetobacter* spp., the gold standard semi-quantitative TCP method was performed as previously described[18,19]. Bacterial suspensions were prepared by inoculating 4–5 colonies from fresh MacConkey agar plate cultures into 10 ml sterile trypticase soy broth (TSB) (Oxoid, Hampshire, UK), and incubated overnight at 37°C. The turbidity was adjusted to match the 0.5 McFarland standard, then diluted 1:100 with TSB. Using a 96-well flat-bottomed sterile tissue culture plate (Nunc, Roskilde, Denmark), 200 µL of the prepared cultures were dispensed into the wells in triplicate. The strong biofilm-forming strain *P. aeruginosa* ATCC 27853 was tested in triplicate and served as a positive control, while eight wells containing sterile TSB served as a negative control. After overnight incubation at 37°C, the well contents were washed three times with 200 µl of phosphate buffer saline (PBS, pH = 7.2, Invitrogen, USA), then fixed with 200 µL of 99% methanol for 15 min. The plates were decanted, air-dried, and then stained with 200 µl of 2% crystal violet (Merk, Germany) for 7 min. Lastly, the dye attached to the fixed cells was dissolved with 160 µl of 33% glacial acetic acid. The optical density (OD) of the stained adherent biofilm was measured at a wavelength of 570 nm using micro-ELISA autoreader Stat Fax-2100 (Awareness Technology, US). The results were averaged and interpreted according to the classification recommended by Babapour *et al.*[19] (Table 1).

Table 1: Interpretation of biofilm formation using tissue culture plate (TCP) method[19]

Mean OD value	Degree of Adherence	Biofilm formation
OD ≤ ODc*	−	None
ODc < OD ≤ 2 ODc	+	Weak
2 ODc < OD ≤ 4 ODc	++	Moderate
4 ODc < OD	+++	Strong

*Optical density cut-off value (ODc) was considered as three standard deviations above the mean optical density (OD) of the negative control

Statistical analysis:

All statistical calculations were done using the Statistical Package for the Social Sciences (SPSS) version 25 for Microsoft Windows. The quantitative data were statistically described in terms of mean ± standard deviation (± SD) for normally distributed data, while the median and interquartile range (IQR) were used for non-normally distributed data. The frequency (count) and relative frequency (percentage) were applied for categorical data. The quantitative variables were compared using the parametric one-way ANOVA, Student's t-tests, and the non-parametric Mann-Whitney U test. To compare categorical variables, the chi-square ($\chi 2$) test was utilized, but Fisher's exact test was used instead when the predicted cell count was less than 5. A statistical significance was determined by a *P* value of ≤ 0.05.

RESULTS

The study involved 50 *Acinetobacter* isolates that were recovered from 28 male and 22 female patients; their ages varied from 23 to 83 years (mean, 52.58 ± 16.81 years), of them 23 (46%) patients died during their ICU stay. The isolates were most frequently obtained from respiratory samples (27/50, 54%), followed by urine (12/50, 24%) and wound swabs (11/50, 22%). Table 2 presents the demographic and clinical characteristics of patients in relation to the clinical outcome. Pneumonia and UTIs were more commonly encountered among the survivors (48.1 % and 22.2 %, respectively) compared to non-survivors (8.7% and 0.0%, respectively), and this was statistically significant (p = 0.002 and 0.025, respectively). In contrast, skin and soft tissue infections were more frequently detected among non-survivors (30.4 % *vs.* 0.0 %, p = 0.002). Regarding admission diagnosis, heart failure was commonly recorded among the survivors (18.5% *vs.* 0.0%, p = 0.054), whereas trauma at admission was significantly higher among the non-survivors (30.4% *vs.* 3.7%, p = 0.017) (Table 2).

Table 2: Demographic and clinical characteristics of the studied patients in relation to the clinical outcome. Data are presented as mean ± standard deviation or number (percentage)

Variables	Clinical outcome			
	Total N = 50	Survived N = 27 (54)	Died N = 23 (46)	*p*-value[a]
Age in years (mean ± SD)	52.58 ±16.81	49.52 ± 13.67	56.17 ± 19.58	0.108[b]
Type of infection				
Pneumonia	15 (30.0)	13 (48.1)	2 (8.7)	**0.002**[c]
VAP	12 (24.0)	4 (14.8)	8 (34.8)	0.099[c]
UTI	6 (12.0)	6 (22.2)	0 (0.0)	**0.025**
Catheter-associated UTIs	6 (12.0)	3 (11.1)	3 (13.0)	1
Skin and soft tissue infections	7 (14.0)	0 (0.0)	7 (30.4)	**0.002**
SSIs	4 (8.0)	1 (3.7)	3 (13.0)	0.322
Admission diagnosis				
Renal failure	8 (16.0)	5 (18.5)	3 (13.0)	0.711
Heart failure	5 (10.0)	5 (18.5)	0 (0.0)	**0.054**
Stroke	7 (14.0)	4 (14.8)	3 (13.0)	1
Active malignancy	2 (4.0)	0 (0.0)	2 (8.7)	0.207
Trauma	8 (16.0)	1 (3.7)	7 (30.4)	**0.017**
Sepsis	6 (12.0)	1 (3.7)	5 (21.7)	0.082
DKA	6 (12.0)	5 (18.5)	1 (4.3)	0.199

Abbreviations: VAP, ventilator-associated pneumonia; UTIs, urinary tract infections; SSIs, Surgical site infection; DKA, diabetic ketoacidosis.
[a] Fisher's Exact test except where specified.
[b] Student's t-test
[c] Pearson Chi-square test
A *p*-value ≤ 0.05 is considered significant.

Antibiotic susceptibility profiles of nosocomial *Acinetobacter* isolates:

Disk diffusion method revealed a higher resistance rate for ciprofloxacin (48/50, 96%), followed by piperacillin (47/50, 94%), ceftriaxone, cefepime (45/50, 90% each), and gentamicin (86%), whereas TMP-SMX displayed the lowest resistance rate (52%) (Figure 1). Surprisingly, all *Acinetobacter* isolates (100%) were resistant to polymyxin B using the BMD method, with minimum inhibitory concentration (MIC) that ranged from 4 to 32 µg/ml. Out of the 50 studied *Acinetobacter* isolates, XDR was detected in 41 (82%) isolates, while eight (16%) isolates were considered MDR. Only one (2%) isolate showed a susceptible (non-MDR/XDR) pattern.

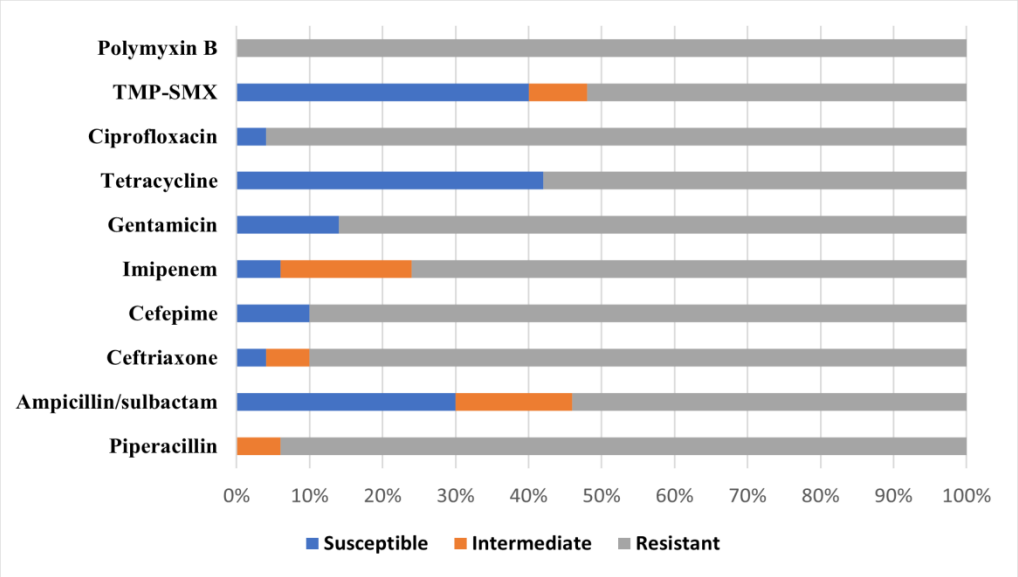

Fig. 1: Antimicrobial susceptibility profile of the studied *Acinetobacter* nosocomial isolates**.** TMP-SMX, trimethoprim-sulfamethoxazole.

Results of biofilm formation among *Acinetobacter* isolates:

The mean OD_{570} (± SD) of the reference strain *P. aeruginosa* ATCC 27853 (positive control) and sterile TSB medium (negative control) was 0.326 ± 0.059 and 0.018 ± 0.004, respectively. Based on the OD_{570} measurements and the ODc calculations, the adherence ability of each *Acinetobacter* isolate was categorized as none, weak, moderate, or strong adherent cells (Figure 2). The strong biofilm formers had a median OD_{570} and IQR value of 0.160 (0.139–0.263), whereas those of moderate biofilm formers were 0.111 (0.095–0.149), and those of weak biofilm formers were 0.058 (0.038–0.082). Among the 50 studied *Acinetobacter* isolates, 49 (98%) were biofilm formers, while a single (2%) isolate was non-biofilm former, with an OD_{570} equal to 0.026. The strong biofilm formers were detected in 38.8% (19/49) of the biofilm-forming isolates, whereas the moderate and weak biofilm formers were detected in 53.1% (26/49) and 8.1% (4/49), respectively. Most biofilm-forming isolates were recovered from sputum (15/49, 30.6%) followed by urine (12/49, 24.5%), while isolates from ETA and wound swabs were equal (11/49, 22.4% each). Of note, the only non-biofilm-forming isolate was recovered from ETA.

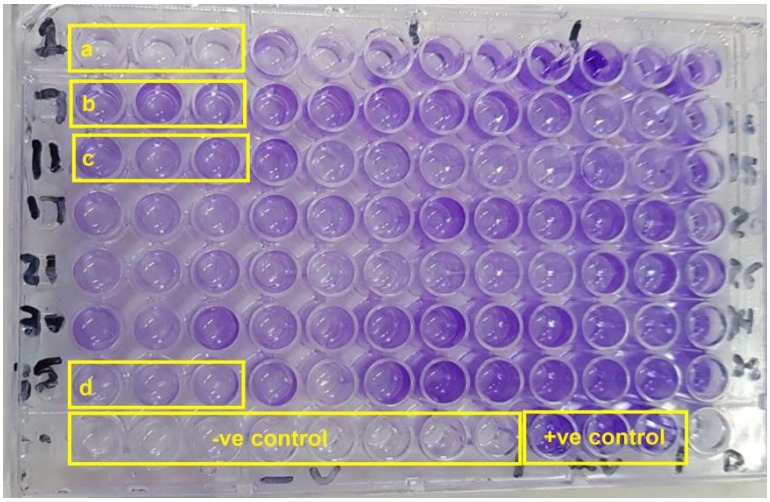

Fig. 2: Tissue culture plate (TCP) method for detection of biofilm formation among *Acinetobacter* clinical isolates. a) A non-biofilm-forming isolate (wells no. A1−A3). b) A strong biofilm-forming isolate (wells no. B1−B3). c) A moderate biofilm-forming isolate (wells no. C1−C3). d) A weak biofilm-forming isolate (wells no. G1−G3). Wells no. H1−H8 was the negative control (sterile trypticase soy broth), while wells no. H9−H11 was the positive control (*P. aeruginosa* ATCC 27853).

The strong biofilm formers were mostly derived from patients with pneumonia (42.1%), the moderate biofilm formers were encountered equally in patients with pneumonia, ventilator-associated pneumonia (VAP), and catheter-associated UTIs (CAUTIs) (23.1%, each), while only two weak biofilm formers were retrieved from VAP patients (50%). Statistically, no significant association was found between them (Table 3). When comparing the different degrees of biofilm formation, it was found that all the isolates obtained from patients with CAUTIs were moderate biofilm formers (6/6, 100%), with a statistically significant difference between them ($p = 0.045$).

Table 3: Distribution of biofilm-forming *Acinetobacter* isolates in relation to the age of patients, the type of infection, and antibiotic resistance profiles. Data are presented as mean ± standard deviation or number (percentage)

Variables	Biofilm-forming *Acinetobacter* isolates				
	Total N = 49	**Strong N = 19 (38.8)**	**Moderate N = 26 (53.1)**	**Weak N = 4 (8.1)**	***p*-value[a]**
Age in years (mean ± SD)	52.57 ± 16.98	53.11 ± 13.99	51.42 ± 20.09	57.50 ± 5.45	0.796[b]
Type of infection					
Pneumonia	15 (30.6)	8 (42.1)	6 (23.1)	1 (25.0)	0.377
VAP	11 (22.4)	3 (15.8)	6 (23.1)	2 (50.0)	0.284
UTI	6 (12.2%)	1 (5.3)	4 (15.4)	1 (25.0)	0.240
Catheter-associated UTIs	6 (12.2)	0 (0.0)	6 (23.1)	0 (0.0)	**0.045**
Skin and soft-tissue infections	7 (14.3)	4 (21.1)	3 (11.5)	0 (0.0)	0.581
SSIs	4 (8.2)	3 (15.8)	1 (3.8)	0 (0.0)	0.505
Antibiotic resistance profiles					
Piperacillin	46 (93.9)	18 (94.7)	24 (92.3)	4 (100)	1
Ampicillin/sulbactam	26 (53.1)	6 (31.6)	17 (65.4)	3 (75.0)	0.144
Ceftriaxone	44 (89.8)	17 (89.5)	23 (88.5)	4 (100)	0.672
Cefepime	44 (89.8)	17 (89.5)	23 (88.5)	4 (100)	1
Imipenem	37 (75.5)	12 (63.2)	21 (80.8)	4 (100)	0.223
Gentamicin	42 (85.7)	17 (89.5)	22 (84.6)	3 (75.0)	0.7
Tetracycline	28 (57.1)	11 (57.9)	14 (53.8)	3 (75.0)	0.835
Ciprofloxacin	47 (95.9)	18 (94.7)	25 (96.2)	4 (100)	1
TMP-SMX	26 (53.1)	13 (68.4)	12 (46.2)	1 (25.0)	**0.048**
Polymyxin B	49 (100)	19 (100)	26 (100)	4 (100)	NA

Abbreviations: VAP, ventilator-associated pneumonia; UTIs, urinary tract infections; SSIs, Surgical site infections; TMP-SMX, trimethoprim-sulfamethoxazole; NA, not applicable.
[a] Fisher's Exact test except where specified. [b]One-way ANOVA test
A p-value ≤ 0.05 is considered significant

Regarding the antibiotic resistance profile, the biofilm-forming isolates exhibited high resistance rates (>80%) to the commonly used antibiotics: polymyxin B (100%), ciprofloxacin (95.9%), piperacillin (93.9%), ceftriaxone and cefepime (89.8%, each), and gentamicin (85.7%) (Table 3). The strong biofilm formers displayed a significantly higher resistance rate (68.4%) to TMP-SMX compared to the moderate and weak biofilm formers (46.2% *vs.* 25%, p = 0.048). Noteworthy, the single non-biofilm-forming isolate was resistant to all tested antibiotics, except TMP-SMX, and thus categorized as an XDR isolate.

Relationship between antibiotic resistance patterns and the strength of biofilm formation:

First, a Mann-Whitney *U* test was used to determine if there is a difference between the MDR and XDR groups regarding the strength of biofilm formation. The MDR isolates displayed higher OD values (median = 0.158, IQR = 0.142−0.193) compared to XDR isolates (median = 0.122, IQR = 0.092−0.167). However, this difference was not statistically significant (p = 0.062) (Table 4, Figure 3). Then, we analyzed the frequency of biofilm-forming groups among MDR and XDR isolates and found that 53.7% and 34.1% of the XDR isolates and 37.5% and 62.5% of the MDR isolates were moderate and strong biofilm formers, respectively. Additionally, none of the MDR isolates displayed a weak biofilm-forming ability (Table 4). Of note, only one isolate of the moderate biofilm formers was neither MDR nor XDR i.e., susceptible, representing 2% of total isolates. When comparing XDR and MDR isolates, the proportion of strong biofilm formers was nearly two times higher in MDR isolates than in XDR isolates (62.5% *vs.* 34.1%). However, no statistically significant difference was observed between the antimicrobial resistance pattern and the strength of biofilm formation (P = 0.442).

Table 4: Relationship between the antibiotic resistance patterns and biofilm-forming ability in *Acinetobacter* nosocomial isolates. Data are presented as median (interquartile range, IQR) or number (percentage)

Parameter	The pattern of resistance of *Acinetobacter* isolates			
	Total N = 50*	**MDR N = 8 (16)**	**XDR N = 41 (82)**	***p*-value**
OD_{570}	0.136 (0.097−0.172)	0.158 (0.142−0.193)	0.122 (0.092−0.167)	0.062[a]
Biofilm-forming				
Strong	19 (38)	5 (62.5)	14 (34.1)	0.442[b]
Moderate	26 (52)	3 (37.5)	22 (53.7)	
Weak	4 (8)	0 (0.0)	4 (9.8)	
Non-biofilm-forming	1 (2)	0 (0.0)	1 (2.4)	

Abbreviations: MDR, multi-drug resistance; XDR, extensive drug resistance; OD_{570}, optical density at 570 nm.
*A single moderate biofilm-former isolate showed a susceptible (non-MDR/XDR) pattern.
[a]Mann-Whitney *U* test [b]Fisher's Exact test
A p-value ≤ 0.05 is considered significant.

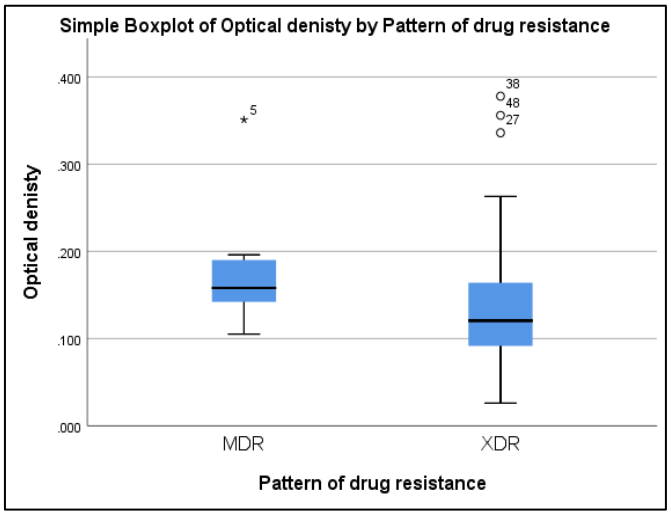

Fig. 3: Box-and-whisker plots present the OD_{570} measurements for both MDR and XDR isolates. Isolates with a higher degree of resistance displayed weaker biofilm formation, as reflected by the lower OD_{570} values. The solid horizontal lines indicate the median value, the box represents the 25% and 75% interquartile ranges, and the whiskers represent minimum and maximum values. Abbreviations: MDR, multidrug resistance; XDR, extreme drug resistance.

DISCUSSION

Acinetobacter is an opportunistic pathogen that is often associated with a wide spectrum of nosocomial infections, especially in critically ill patients[19]. In the current study, the majority of *Acinetobacter* isolates were obtained from lower respiratory tract samples (27/50, 54%), while isolates from wound swabs were the lowest (11/50, 22%). This finding agrees with previous studies that showed maximum isolation of *Acinetobacter* spp. from respiratory samples[12,20]. Nevertheless, Dash *et al.*[21] found a higher rate of isolation from pus and wound swabs (56%). The differences in the isolation rate of *Acinetobacter* strains from different clinical samples could be elucidated by the considerable variation in the frequency of infections in different populations and clinical settings.

In our cohort, most of the mortality was observed in patients with VAP (34.8%). This finding seems in line with an Egyptian study conducted at Tanta University Hospital which revealed 41.4% (12/29) of mortality cases were attributed to *A. baumannii*, with VAP identified as a prominent contributing risk factor, accounting for 72.4% of the cases[22]. An additional study reported a comparable mortality rate (37.2%, 29/78) in ICU patients with VAP caused by *A. baumannii*[23]. Despite being an uncommon presentation, *A. baumannii* has evolved as a serious cause of skin and soft tissue infections that can lead to high mortality rates[24,25]. Our finding agrees with these studies, as 30.4% of deaths were detected in patients with skin and soft tissue infections. It is worth mentioning that none of the patients who had UTIs died during our study. Similarly, a matched cohort analysis of the incidence and attributable mortality of healthcare-associated infections in European ICUs from 2008 to 2012[26] reported that patients with UTIs did not have a higher mortality rate.

In the present study, *Acinetobacter* isolates showed a high resistance rate to ciprofloxacin (96%), piperacillin (94%), ceftriaxone and cefepime (90% each), and gentamicin (86%), whereas TMP-SMX demonstrated the lowest resistance rate (52%). Consistent with our findings, Lasheen *et al.*[12] reported a higher resistance rate of *Acinetobacter* isolates to cefepime (88%), ceftriaxone, and piperacillin (86% each), with > 50% resistance to gentamycin, amikacin, ceftazidime, and ciprofloxacin. In a Saudi Arabian study[27], *Acinetobacter* spp. exhibited a higher resistance rate against levofloxacin (83.3%), along with resistance rates ranging from 61.2% to 72.3% for all tested cephalosporins, carbapenems, and aminoglycosides, while the resistance rate for TMP-SMX was 55.6%. In a different study, it was observed that 100% of the test isolates were resistant to piperacillin, ceftazidime, cefepime, and aztreonam, with a 94.5% resistance rate to ciprofloxacin that is consistent with our study[20]. The high level of antibiotic resistance observed in our study could be linked to the selective pressure exerted by the frequent use of these drugs in our hospitals, as well as the ability of *Acinetobacter* to rapidly acquire resistance genes through horizontal gene transfer.

Our study showed that all isolates (100%) were resistant to polymyxin B, with MIC values ranged from 4 to 32 µg/ml. A similar rate was reported by a previous study that used colistin for susceptibility testing[28]. Lower rates were reported in Italy (69%)[29], Greece (29%)[30], Saudi Arabia (5.5%)[27], and Egypt (5%)[31], whereas other studies demonstrated 100% susceptibility to polymyxins[20,21]. The high rate of resistance to polymyxin B in our study may be attributed to the increased use of polymyxin B in the treatment of carbapenem-resistant *Acinetobacter* infections, particularly in ICUs where bacterial selective pressure is frequently high. Moreover, a monotherapy approach with polymyxin B requires caution due to the lack of consistently effective plasma concentrations, as evidenced by the evolving pharmacodynamic and pharmacokinetic data[32]. Therefore, combination therapy with polymyxin may enhance antimicrobial activity and prevent the development of resistance[33].

The incidence rate of XDR and MDR isolates in our study was 82% and 16%, respectively. Similarly, a study from Egypt noticed that 91.7% of isolates were XDR and 8.3% of them were MDR[20]. Another study conducted by Khalifa *et al.*[27] revealed that 50% of *Acinetobacter* isolates were XDR and 16.6% were MDR. It seems that the emergence of XDR and MDR *Acinetobacter* strains in our hospital is strongly linked to the selective pressure caused by the prolonged use of broad-spectrum antimicrobials, thus limiting the therapeutic strategies available to control infections.

Our results revealed that 49 (98%) of *Acinetobacter* isolates were biofilm-forming. Consistent with our findings, Nahar *et al.*[34] noticed that the biofilm-forming *Acinetobacter* spp. was substantially high, accounting for 87.5% of the total ICU *Acinetobacter* isolates. In our work, the moderate biofilm formers (53.1%) predominated over the strong and weak biofilm formers (38.8% and 8.1%, respectively). A similar study carried out on *A. baumannii* reported that the moderate biofilm formers (82/155, 52.9%) were the most prevalent, with 21.3% and 25.8% being strong and weak biofilm formers, respectively[19]. Another study in Egypt reported that 34% of biofilm-forming isolates were moderate formers, while the strong and weak formers comprised 20.2% and 16%, respectively[7]. By contrast, Shenkutie *et al.*[14] found that the highest number of isolates produced biofilm strongly (25%) compared to the moderate and weak biofilm producers (14.4% and 20.2%, respectively). The difference in the incidence rate of biofilm grading observed across various studies may be related to the varying number of clinical isolates obtained from distinct sources[10].

Despite previous studies have found a significant association of biofilm-forming *Acinetobacter* with device-associated respiratory infections[34,35], our study revealed that biofilm-forming *Acinetobacter* was more frequently isolated from patients with pneumonia (30.6%), followed by VAP (22.4%). Likewise, an earlier study reported that 30% of biofilm-producing isolates were retrieved from pneumonia patients[36]. Interestingly, we found that all cases with CAUTIs (100%, $p = 0.045$) are infected with moderate biofilm formers, however, this significant association was not detected in samples originating from other sources. Our finding seems to suggest that moderate biofilm formers could achieve a balance between persistence and virulence without causing acute complications. Strong biofilm formers can form a dense and impenetrable biofilm that obstructs the catheter lumen, whereas weak biofilm formers are more susceptible to clearance, limiting their persistence and chronicity.

In the present study, the biofilm-forming isolates showed high resistance rates to polymyxin B (100%), ciprofloxacin (95.9%), piperacillin (93.9%), ceftriaxone and cefepime (89.9%, each), and gentamicin (85.7%). A comparable study showed that all (100%) biofilm-producing *Acinetobacter* spp. were resistant to amoxicillin, ceftriaxone, ceftazidime, cefotaxime, cefuroxime, gentamicin, 82.1% were resistant to ciprofloxacin, and only 7.1% of them were resistant to colistin[34]. The discrepancy in findings between the present study and other researchers can be attributed to the wide variation in the pattern of resistance exhibited by hospital-acquired bacterial strains among different countries and even over time within a single country[10]. It was observed in this work that the strong biofilm formers exhibited a significantly higher resistance rate (68.4%) to TMP-SMX than the moderate (46.2%) and weak (25.0%) biofilm formers. Similarly, it has been shown that *E. coli* strains with strong biofilm formation ability display higher resistance to cotrimoxazole, amoxicillin-clavulanic acid, norfloxacin, gatifloxacin, and gentamicin[37]. The reason behind this could be related to the inability of these antibiotics to penetrate the EPS matrix produced by the strong biofilm formers. Moreover, this finding highlights the importance of considering the strength of biofilm formation when selecting antibiotics for the effective treatment of bacterial infections.

This study noticed that the strong biofilm-forming group among MDR isolates was approximately two times higher than among XDR isolates (62.5% *vs.* 34.1%, $P = 0.442$). In agreement with our findings, several earlier studies reported an inverse relationship between the strength of biofilm formation in *A. baumannii* and the development of MDR/XDR patterns, with weaker biofilm formation among isolates with a higher level of resistance[14,35,38]. Although the precise mechanisms underlying this phenomenon are not yet fully understood, it is speculated that the genetic mechanisms responsible for antibiotic resistance could potentially reduce the ability of microorganisms to form biofilm. It has been confirmed that horizontal transfer of β-lactamase resistance genes can hinder biofilm formation in *E. coli* and *P. aeruginosa* by disrupting the cell adhesion processes required to initiate biofilm formation[39]. Another possible explanation is the diverse mechanisms of antibiotic resistance exhibited by strains with no or weak biofilm formation[13]. However, other studies indicated that stronger biofilm formation may be correlated with a broader antimicrobial susceptibility pattern[3,11,40]. Our study could not establish a statistically significant association between the strength of biofilm formation by *Acinetobacter* strains and the pattern of resistance; this could be attributed to the small sample size; therefore, we recommend conducting further investigations using a larger sample size and additional studies based on molecular levels to settle or reject the association.

CONCLUSIONS

This study concluded that biofilm formation was highly prevalent among the nosocomial isolates of *Acinetobacter* spp., which could increase the colonization of MDR and XDR bacteria in hospital settings, particularly in ICUs. Analysis of the results of this study showed an inverse relationship between the development of MDR/XDR patterns and the strength of biofilm formation of *Acinetobacter* isolates. These findings indicate that biofilm formation is an important survival mechanism of *Acinetobacter* nosocomial isolates that have an insufficient level of antibiotic resistance. This necessitates finding effective preventive measures against biofilm formation and the appliance of proper infection control measures in ICU settings.

Consent for publication:
Not applicable
Availability of data and material:
All datasets generated during and/or analyzed during the current study were presented in the main article and are available from the corresponding author upon reasonable request.
Competing interests:
The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
Funding:
The authors did not receive any funds or grants from funding agencies.

Authors' contributions:
Shaimaa Mohamed Seleim: material preparation, performance of the experiments, data collection, and analysis. Mona Abd El-Wahab Abd El-Massih:

supervision and revision of the manuscript. Mohamed Hamdi Saleh: supervision, data analysis, and revision of the manuscript. Doaa Mahdy El-Wakil: providing the study conception and design, supervision, data analysis, and writing the original draft of the manuscript. All authors provided critical feedback and took part in reviewing and revising the intellectual as well as the technical parts of the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Engür D, Çetinkaya Çakmak B, Kaynak Türkmen M, Telli M, Eyigör M, Güzünler M. A milk pump as a source for spreading *Acinetobacter baumannii* in a neonatal intensive care unit. Breastfeed Med 2014;9(10):551−4. http://doi.org/10.1089/bfm.2014.0054
2. Anbazhagan D, Mansor M, Yan GO, Md Yusof MY, Hassan H, Sekaran SD. Detection of quorum sensing signal molecules and identification of an autoinducer synthase gene among biofilm forming clinical isolates of *Acinetobacter* spp. PloS one 2012;7(7):e36696. https://doi.org/10.1371/journal.pone.0036696
3. 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;19:629. https://doi.org/10.1186/s12879-019-4272-0
4. Weber DJ, Rutala WA, Miller MB, Huslage K, Sickbert-Bennett E. Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, *Clostridium difficile*, and *Acinetobacter* species. Am J Infect Control 2010;38(5):S25−S33. https://doi.org/10.1016/j.ajic.2010.04.196
5. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18(3):268−81. https://doi.org/10.1111/j.1469-0691.2011.03570.x
6. CDC, Antibiotic Resistance Threats in the United States. Centers for Disease Control and Prevention, Atlanta, GA, U.S. Department of Health and Human Services, 2019. http://www.cdc.gov/drugresistance/Biggest-Threats.html
7. 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. https://doi.org/10.2147/IDR.S261939
8. M'hamedi I, Hassaine H, Bellifa S, Lachachi M, Terki IK, Djeribi R. Biofilm formation by *Acinetobacter baumannii* isolated from medical devices at the intensive care unit of the University Hospital of Tlemcen (Algeria). Afr J Microbiol Res 2014;8(3):270−6. https://doi.org/10.5897/AJMR2013.6288
9. Krzyściak P, Chmielarczyk A, Pobiega M, Romaniszyn D, Wójkowska-Mach J. *Acinetobacter baumannii* isolated from hospital-acquired infection: biofilm production and drug susceptibility. APMIS 2017;125(11):1017−26. https://doi.org/10.1111/apm.12739
10. Abdi-Ali A, Hendiani S, Mohammadi P, Gharavi S. Assessment of biofilm formation and resistance to imipenem and ciprofloxacin among clinical isolates of *Acinetobacter baumannii* in Tehran. Jundishapur J Microbiol 2014;7(1):e8606. https://doi.org/10.5812/jjm.8606
11. Yang CH, Su PW, Moi SH, Chuang LY. Biofilm formation in *Acinetobacter baumannii*: genotype-phenotype correlation. Molecules 2019;24(10):1849. https://doi.org/10.3390/molecules24101849
12. Lasheen A, Sleem A, Elmahdy E. Multi-drug resistance profile of virulent biofilm forming *Acinetobacter baumannii* isolates in intensive care units. Egypt J Med Microbiol 2023; 32(1):105−11. doi: 10.21608/ejmm.2023.277786
13. Rahimi S, Farshadzadeh Z, Taheri B, Mohammadi M, Haghighi MA, Bahador A. The relationship between antibiotic resistance phenotypes and biofilm formation capacity in clinical isolates of *Acinetobacter baumannii*. Jundishapur J Microbiol 2018;11(8):e74315 https://doi.org/10.5812/jjm.74315
14. Shenkutie AM, Yao MZ, Siu GK, Wong BK, Leung PH. Biofilm-induced antibiotic resistance in clinical *Acinetobacter baumannii* isolates. Antibiotics 2020;9(11):817. https://doi.org/10.3390/antibiotics9110817
15. Rhodes A, Evans LE, Alhazzani W et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Intensive Care Med 2017;43:304–77. https://doi.org/10.1007/s00134-017-4683-6
16. Cheesbrough M. Microbiological tests. In: Direct Laboratory Practice in Tropical Countries, Part 2, 2nd edition. Cambridge University Press; 2009, pp. 1−266.

17. CLSI, Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. CLSI supplement M100. Wayne, PA, USA. Clinical and Laboratory Standards Institute, 2018. https://clsi.org/media/1930/m100ed28_sample.pdf
18. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985;22(6):996−1006. https://doi.org/10.1128/jcm.22.6.996-1006.1985
19. Babapour E, Haddadi A, Mirnejad R, Angaji SA, Amirmozafari N. Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. Asian Pac J Trop Biomed 2016;6(6):528−33. https://doi.org/10.1016/j.apjtb.2016.04.006
20. Ibrahim M, Abdallah S, Soliman I, Afifi N, Atiat Allah M. Hospital-acquired infection by carbapenem-resistant *Acinetobacter* species in ICUs in Assiut, Egypt. Egypt J Med Microbiol 2023;32(1):97−104. doi: 10.21608/ejmm.2023.277785
21. Dash M, Padhi S, Pattnaik S, Mohanty I, Misra P. Frequency, risk factors, and antibiogram of *Acinetobacter* species isolated from various clinical samples in a tertiary care hospital in Odisha, India. Avicenna J Med 2013;3(04):97−102. https://doi.org/10.4103/2231-0770.120501
22. Al-Hassan LL, Al-Madboly LA. Molecular characterisation of an *Acinetobacter baumannii* outbreak. Infect Prev Pract 2020;2(2):100040.
23. Ju M, Hou D, Chen S, Wang Y, Tang X, Liu J, Chen C, Song Y, Li H. Risk factors for mortality in ICU patients with *Acinetobacter baumannii* ventilator-associated pneumonia: impact of bacterial cytotoxicity. J Thorac Dis 2018;10(5):2608−17. doi: 10.21037/jtd.2018.04.86
24. Ali A, Botha J, Tiruvoipati R. Fatal skin and soft tissue infection of multidrug resistant *Acinetobacter baumannii*: a case report. Int J Surg Case Rep 2014;5(8):532−6. https://doi.org/10.1016/j.ijscr.2014.04.019
25. Guerrero DM, Perez F, Conger NG, Solomkin JS, Adams MD, Rather PN, Bonomo RA. *Acinetobacter baumannii*-associated skin and soft tissue infections: recognizing a broadening spectrum of disease. Surg Infect 2010;11(1):49−57. https://doi.org/10.1089/sur.2009.022
26. ECDC, Incidence and attributable mortality of healthcare-associated infections in intensive care units in Europe, 2008−2012. European Centre for Disease Prevention and Control, Stockholm, 2018. https://www.ecdc.europa.eu/en/publications-data/incidence-and-attributable-mortality-healthcare-associated-infections-intensive
27. Khalifa R, Aljahdali H, Alghamdi H, Ismail B, Joharjy H, Eibani K. Characterization of β-lactamase genes among multidrug- and extensively drug-resistant *Acinetobacter* and *Enterobacteriaceae* species causing infections in critically ill patient. Egypt J Med Microbiol 2023; 32(1): 113−23. doi: 10.21608/ejmm.2023.277787
28. Samonis G, Matthaiou DK, Kofteridis D, Maraki S, Falagas ME. *In vitro* susceptibility to various antibiotics of colistin-resistant gram-negative bacterial isolates in a general tertiary hospital in Crete, Greece. Clin Infect Dis 2010;50(12):1689−91. https://doi.org/10.1086/653009
29. Agodi A, Voulgari E, Barchitta M, Quattrocchi A, Bellocchi P, Poulou A, Santangelo C, Castiglione G, Giaquinta L, Romeo MA, Vrioni G. Spread of a carbapenem-and colistin-resistant *Acinetobacter baumannii* ST2 clonal strain causing outbreaks in two Sicilian hospitals. J Hosp Infect 2014;86(4):260−6. https://doi.org/10.1016/j.jhin.2014.02.001
30. Mavroidi A, Likousi S, Palla E, Katsiari M, Roussou Z, Maguina A, Platsouka ED. Molecular identification of tigecycline-and colistin-resistant carbapenemase-producing *Acinetobacter baumannii* from a Greek hospital from 2011 to 2013. J Med Microbiol 2015;64(9):993−7. https://doi.org/10.1099/jmm.0.000127
31. Abdulzahra AT, Khalil MA, Elkhatib WF. First report of colistin resistance among carbapenem-resistant *Acinetobacter baumannii* isolates recovered from hospitalized patients in Egypt. New Microbes New Infect 2018;26:53−8. https://doi.org/10.1016/j.nmni.2018.08.007
32. Sandri AM, Landersdorfer CB, Jacob J, Boniatti MM, Dalarosa MG, Falci DR, Behle TF, Bordinhão RC, Wang J, Forrest A, Nation RL. Population pharmacokinetics of intravenous polymyxin B in critically ill patients: implications for selection of dosage regimens. Clin Infect Dis 2013;57(4):524−31. https://doi.org/10.1093/cid/cit334
33. Garonzik SM, Li J, Thamlikitkul V, Paterson DL, Shoham S, Jacob J, Silveira FP, Forrest A, Nation RL. Population pharmacokinetics of colistin methanesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients. Antimicrob Agents Chemother 2011;55(7):3284−94. https://doi.org/10.1128/aac.01733-10
34. Nahar A, Anwar S, Miah MRA. Association of biofilm formation with antimicrobial resistance

among the *Acinetobacter* species in a tertiary care hospital in Bangladesh. Journal of Medicine 2013;14(1):28−32. https://doi.org/10.3329/jom.v14i1.14533

35. Alamri AM, Alsultan AA, Ansari MA, Alnimr AM. Biofilm-formation in clonally unrelated multidrug-resistant *Acinetobacter baumannii* isolates. Pathogens 2020;9(8):630. https://doi.org/10.3390/pathogens9080630
36. Rao RS, Karthika RU, Singh SP, Shashikala P, Kanungo R, Jayachandran S, Prashanth K. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acinetobacter baumannii*. Indian J Med Microbiol 2008;26(4):333−7. https://doi.org/10.1016/S0255-0857(21)01809-0
37. Mittal S, Sharma M, Chaudhary U. Biofilm and multidrug resistance in uropathogenic *Escherichia coli*. Pathog Glob Health 2015;109(1):26−9. https://doi.org/10.1179/2047773215Y.0000000001
38. Li Z, Ding Z, Liu Y, Jin X, Xie J, Li T, Zeng Z, Wang Z, Liu J. Phenotypic and genotypic characteristics of biofilm formation in clinical isolates of *Acinetobacter baumannii*. Infect Drug Resist 2021;14:2613−24. https://doi.org/10.2147/IDR.S310081
39. Gallant CV, Daniels C, Leung JM, Ghosh AS, Young KD, Kotra LP, Burrows LL. Common β-lactamases inhibit bacterial biofilm formation. Mol Microbiol 2005;58(4):1012−24. https://doi.org/10.1111/j.1365-2958. 2005. 04892.x
40. Bahador A, Farshadzadeh Z, Raoofian R, Mokhtaran M, Pourakbari B, Pourhajibagher M, Hashemi FB. Association of virulence gene expression with colistin-resistance in *Acinetobacter baumannii*: analysis of genotype, antimicrobial susceptibility, and biofilm formation. Ann Clin Microbiol Antimicrob 2018;17:24. https://doi.org/10.1186/s12941-018-0277-6