Egyptian Journal of Medical Microbiology Volume 32 / No.4 / October 2023 1-8 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Insights into Biofilm-based Multidrug Resistance in *Acinetobacter Baumannii* Isolated from Patients Hospitalized in Benha University Intensive Care Unit

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Key words:
Virulence, PCR, Antimicrobial

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ABSTRACT

Background: *Acinetobacter baumannii (A.baumannii) is an opportunistic pathogen that causes serious types of infections in human. It shows a wide range of antibiotic resistance.* ***Objective****: to investigate biofilm formation and its relation to biofilm-related genes in multidrug-resistant (MDR) A.baumannii isolated from patients hospitalized in Benha University Intensive Care Unit (ICU).* ***Methodology:*** *This study was conducted on (90) clinical samples obtained from immunocompromised patients with various symptomatic clinical infections, at least 48 hours after admission to Benha University ICU. A. baumannii isolates were identified, their antibiotic susceptibility pattern was detected using VITEK® 2 system. Biofilm-forming capacity of A. baumannii isolates was determined by tube method. Biofilm-related genes (csuE , omp A, bap , bla $_{PER-1,}$ and aba I) were detected using polymerase chain reaction(PCR).* ***Results:*** *Out of 60 A.baumannii isolates, 93.3% were MDR, 76.7% were extensive drug resistant (XDR), and 75% were biofilm formers. A significant relationship was revealed between the multidrug resistance and biofilm formation. Detected ratio of csuE, omp A, bap, bla $_{PER-1,}$ and aba I genes were 96.7%, 48.3% , 68.3% , 40% and 88.3% respectively. (csu E, bap, aba I, and bla $_{PER-1}$) genes were founded to have a statistically significant relation to biofilm formation. While (omp A) gene was insignificantly related to biofilm formation.* ***Conclusion****: Biofilm formation in A. baumannii is significantly related to multidrug resistance, with high frequency of biofilm -related genes.*

INTRODUCTION

A. baumannii is Gram- negative, aerobic, glucose non-fermentative, non-motile coccobacillus, ubiquitous in nature, and persistent in healthcare settings[1]. *A. baumannii* is regarded as a significant opportunistic human pathogen[2]. It is related to various illnesses, such as peritonitis, bacteremia, meningitis, wound and soft-tissue infections, besides ventilator-associated pneumonia[3].

It has become one of the most problematic microbes causing nosocomial infections due to its remarkable ability to survive in the hospital environment and the rapid development of multiple antibiotic resistance resulting in the emergence of multidrug-resistant strains[4].

One of the important virulence factor of *A. baumannii* is its ability to form biofilm, which is related to its high degree of antibiotic resistance[5]. Biofilms are complex mixtures of microbes which are attached to hard surfaces [6]. They are frequently enclosed in a thick polysaccharide covering, that renders them antibiotic-resistant and difficult to eradicate [6].

Several virulence factors contribute to *A. baumannii 's* biofilm forming ability as; outer membrane protein A (OmpA), biofilm associated protein (Bap), chaperon-usher pilus (CUP), extracellular exopolysaccharide (EPS) , two-component regulatory system (TCRS) (BfmS/BfmR), poly-β-(1,6)-N-acetyl glucosamine (PNAG) and quorum sensing system [7].

The development of pili involved in adhesion to abiotic surfaces requires the presence of *CsuA/BABCDE* gene. Inactivating the *csuE* gene, which is a component of the *CsuA/BABCDE* chaperone-usher complex, has been shown to reduce the production of pili and the development of biofilms [8].

Bap gene is known to significantly impact bacterial aggregation, intercellular adhesion, and the ability to form biofilm[9]. Despite using completely different pathways, *OMPs* in *A. baumannii* serve diverse roles in

promoting the bacterial adaptation to antibiotic- and host-induced stressors [10].

The quorum sensing (QS) system (*abaI*/*abaR*) presented by *A. baumannii* shows an autoinducer synthase (*AbaI*) component, which plays a fundamental role in the production of Acyl-homoserine lactones (AHL). Gram negative bacteria use the autoinducer signals, which contain a significant amount of AHL, to produce biofilms [7].

The current study aimed to investigate the biofilm formation frequency in multidrug -resistant *A. baumannii* isolated from patients hospitalized in Benha University ICU and its relationship to biofilm related genes.

METHODOLOGY

The current cross-sectional study was performed between March 2023 and September 2023 on immunocompromised patients admitted to the ICU of Benha University Hospital. Immunocompromised patients with symptomatic clinical infections at least 48 h after ICU admission were randomly selected as study participants. A written informed consent was obtained from each participant or their legal guardian before inclusion in the study after Benha University Research Ethics Committee authorized it under number (Rc 6.3.2023).

Ninety clinical specimens were collected from patients and cultured on routine bacteriological media including**:** (23) blood, (21) sputum, (15) wound swabs, (17) chest tube secretions and (14) urine samples. Pure isolates were stored at -80°C for further investigations.

Bacterial isolates identification and testing the antimicrobial susceptibility

Identification of *A. baumannii* isolates was done through VITEK®2 microbial identification system (BioM'erieux, France) using VITEK® 2 GN ID cards (BioM'erieux, France) according to the instructions of the manufacturer. AST-GN67 test card (BioM'erieux, France) utilized on VITEK ® 2 system , and antibiotic susceptibility profiles were assessed for each isolate. Minimum inhibitory concentrations (MICs) to twelve antibiotics including ampicillin/ sulbactam , piperacillin/ tazobactam, gentamicin, meropenem, ciprofloxacin, levofloxacin, tobramycin, cefepime, ceftazidime, ceftriaxone, cefazoline and trimethoprim/ sulfamethoxazole, were reported.The breakpoints for interpretation of the antibiotic susceptibility tests as susceptible, intermediate and resistant were in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017) [11].

The definition of MDR is stated as; resistance to at least one antibiotic in three or more groups of antibiotics. However, XDR was defined as acquiring resistance to at least one antibiotic in all but two or fewer antimicrobial classes[12].

Biofilm formation by tube method:

Test tubes containing 10 mL trypticase soy broth (TSB) and 1% glucose were inoculated with a loopful of *A. baumannii*. The inoculated test tubes were incubated for 24 h, at 37 **º**C. Then, the liquid media were decanted. After being washed in phosphate buffered saline (pH 7.3), the bacterial cell pellets were allowed to dry. Tubes were stained with crystal violet (0.1%) for 7 minutes and then rinsed with distilled water for 5 minutes. Tubes were kept in inverted position to dry. A visible film lining the wall and the bottom of the tube indicates positive biofilm formation. Sterile TSB tubes were used as negative control [13].

Detection of Biofilm Related Genes by PCR

- DNA extraction: It was done using ABT Bacterial DNA Mini Extraction Kit according to manufacturer's instructions (Applied Biotechnology, Egypt). Purified DNA stored at -20ºC till used.
- DNA amplification: using 2X TOPsimple™ DyeMIX-nTaq kit (enzynomics, Korea)**.** The procedure followed the manufacturer's instructions. PCR was performed in a thermal cycler (Biometra, Germany).
- Primers used in our study are described in table (1) for *bap, omp A, csuE, bla$_{PER-1}$, and aba I genes.*
- Conditions for the PCR were initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, an annealing temperature for each gene (according to table 1) for 1 min, an extension at 72 °C for 1 min /kb followed by a final extension at 72 °C for 5 min. The amplified products were electrophoresed on 1.5% agarose gel and were visualized by ethidium bromide staining (Fig 1).

Table 1: Primers used in this study for detection of biofilm related genes:

Target Genes	Primers sequences (5–3)	DNA amplicon Size (bp)	Annealing Temp (C°)	Reference
bap	Fw- TGCTGACAGTGACGTAGAACCACA Rv- TGCAACTAGTGGAATAGCAGCCCA	184	54	14
omp A	Fw-CTGGTGTTGGTGCTTTCTGG Rv-GTGTGACCTTCGATACGTGC	352	49	7
csu E	Fw- AGACATGAGTAGCTTTACG Rv- CTTCCCCATCGGTCATTC	516	25	15
bla $_{PER-1}$	Fw- ATGAATGTCATTATAAAAGC Rv- AATTTGGGCTTAGGGCAGAA	925	50.5	16
aba I	Fw-CGCTACAGGGTATTTGTTGA Rv- TCGTAATGAGTTGTTTTGCG	370	46	7

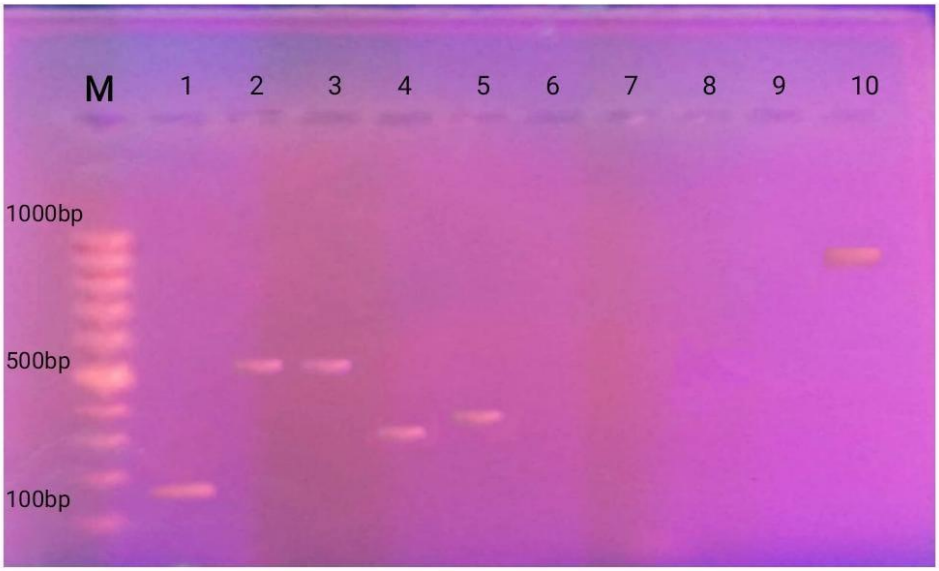

Fig. 1: PCR products of biofilm related genes (*bap* , *omp A, csuE* , *bla* $_{PER-1,}$, and *aba I*) .
Lane M: 100 bp DNA ladder. Lane 1: amplified fragment of *bap* gene (184 bp). Lane 2, 3: amplified fragments of *csuE* gene (516 bp). Lane 4: amplified fragment of *omp A* gene (352 bp). Lane 5: amplified fragment of *aba I* gene (370 bp). Lane 10: amplified fragment of *bla* $_{PER-1}$ gene. (925 bp).

Statistical analysis:

SPSS version 17.0 software (SPSS, Inc., Chicago, IL) was used for data analysis. Statistically significant data were identified using chi-square test. P value of <0.05 is considered significant.

RESULTS

Out of ninety clinical specimens collected from immunocompromised ICU patients with symptomatic clinical infections, (60) *A. baumannii* were recovered. Nineteen (31.7%), 16 (26.7%), 10 (16.7%), 8 (13.3%) and 7 (11.7%) isolates were recovered from blood, sputum, urine, chest tube collected secretions and wound swabs respectively.

Antimicrobial resistance profiles of *A.baumannii* isolates:

Forty-three isolates (71.7%) were resistant to ampicillin/sulbactam , 42 isolates (70 %) to cefotaxime , 40 isolates (66.7%) to cefazolin, 39 isolates (65%) to ciprofloxacin, 35 isolates (58.3%) to ceftriaxone, 32 isolates (53.3%) to trimethoprim/sulfamethoxazole , 28 isolates (46.7%) to meropenem, 26 isolates (43.3%) to piperacillin/tazobactam, 24 isolates (40%) to tobramycin, 19 isolates (31.7%) to levofloxacin, 18 isolates (30%) to cefepime and 18 isolates (30%) to gentamicin (Fig 2).

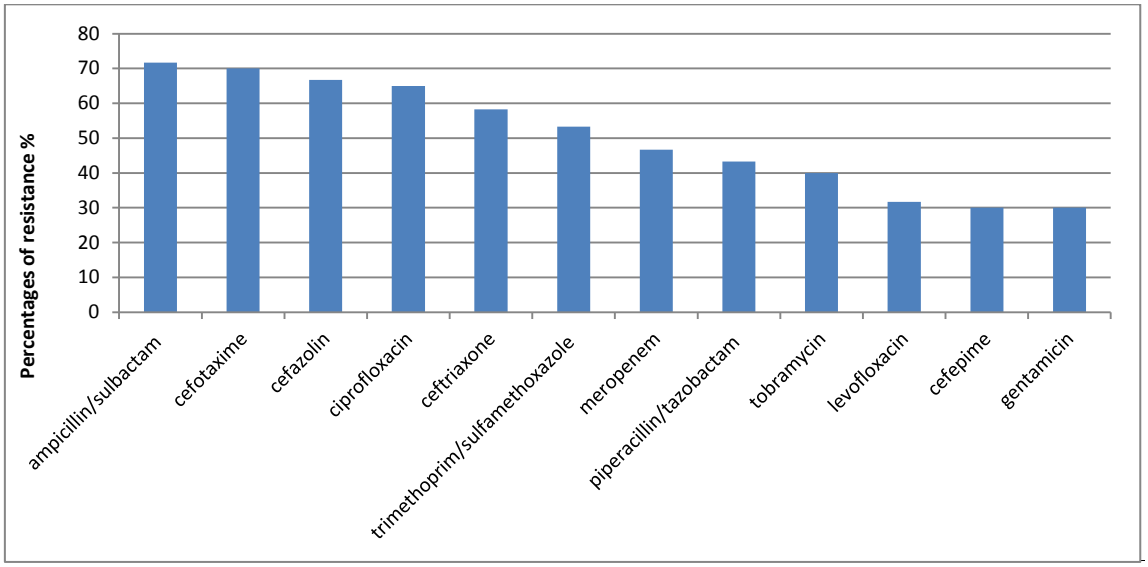

Fig. 2: Antimicrobial resistance profiles of *A.baumannii* isolates .

Fifty-six isolates (93.3%) of tested *A. baumannii* were identified as MDR , however 46 isolates (76 .7%) were XDR.

Biofilm formation:

Of the (60) *A. baumannii* tested for biofilm formation by tube method, 45 isolates (75%) had the power to form biofilm (Fig 3).

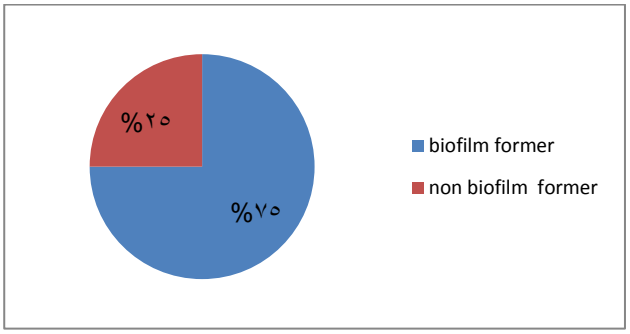

Fig. 3: Percentage of biofilm formation of *A. baumannii* isolates which was detected by tube method.

Multidrug resistance and the biofilm forming ability of *A. baumannii* were analyzed for the relationship between them and it was significant (P value < 0.05) (Table 2).

Table 2: Relationship between multidrug resistance and *A. baumannii* biofilm formation:

Biofilm formation	**MDR**			**P value**
	Positive	Negative	Total	
Positive	44	1	45	<0.05
Negative	12	3	15	
Total	56	4	60	

Detection of Biofilm- Related Genes by PCR:

csu E , omp A , bap, aba I, and bla $_{PER-1}$ genes were tested by PCR giving results shown in (Table 3).

Table 3: Biofilm-related genes frequency in *A. baumannii* isolated from different clinical samples:

Biofilm related Gene	**Number of isolates (%)**
csu E	58 (96.7%)
aba I	53 (88.3%)
bap	41 (68.3%)
omp A	29 (48.3%)
bla $_{PER-1}$	24 (40%)

In table 4, (*csu E , bap, aba I and bla* $_{PER-1}$) genes founded to have a statistically significant relation to biofilm formation (P value < 0.05) . While (*omp A*) gene was insignificantly related to biofilm formation. (P value > 0.05) .

Table 4: Relation between biofilm formation and identified genes :

Biofilm formation	**Biofilm-related genes**				
	csu E	***aba I***	***bap***	***omp A***	***bla*** $_{PER-1}$
Biofilm former	43	43	38	16	22
Non biofilm former	15	10	3	13	2
Total	58	53	41	29	24
P value	<0.05	<0.05	<0.05	>0.05	<0.05

DISCUSSION

A. baumannii easily survive and spread in hospital environment probably due to its ability to form a biofilm[17]. *A. baumannii*, is an opportunistic Gram-negative bacterium with increased clinical importance, due its ability to cause healthcare associated infections in critically ill patients[18]. Amongst the ninety clinical samples collected from the ICU immunocompromised patients, sixty (66.7%) *A. baumannii* isolates were recovered, which is parallel to a study performed by Babapour et al. in 2016[19]. Likewise, another study reported that 71% from a total of 50 clinical samples, were *A baumannii* [20].

In the current study, 19 isolates (31.7%) of *A. baumannii* were recovered from blood samples, 16 isolates (26.7%) from sputum, 10 isolates (16.7%) from urine, 8 isolates (13.3%) from chest tube collected secretions, and 7 isolates (11.7%) from wound swabs. *A. baumannii* was reported to colonize the gastrointestinal tract, urinary tract, conjunctiva, skin, and oral cavities[21], and it is known to cause various infections such as, pneumonia, ventilator-associated pneumonia (VAP), endocarditis, urinary tract infection, bacteremia, skin infections, wound infection, and meningitis [22].

A. baumannii has developed an increased rate of multidrug resistance, which posed a severe threat to the public health[23]. Isolated *A. baumannii* with resistance to at least one antibiotic in three or more antimicrobial categories are considered MDR pathogens, this criterion applies to about 56 isolates (93.3%) of tested *A. baumannii* detected in our study. However, about 46 isolates (76.7%) were considered as XDR. The results of our study are also consistent with other researchers who reported 92.95% of the isolated *A. baumannii* as MDR and 86.53% as XDR[19]. A research of Saadulla and Muhammed , published earlier in 2023 , recorded about 79% of tested *A. baumannii* isolates as MDR [24]. In disagreement with our results, two studies of Soroush et al.[25] and Shali et al.[26] had considered 40.6% and 18.92% of their *A. baumannii* isolates as MDR respectively.

In Egypt, drug resistance had emerged quickly and horribly in the healthcare environment as a result of increased demands and poorly regulated antibiotic use[27]. The results of the present study revealed, resistance to ampicillin/sulbactam (71.7%), cefotaxime (70%), cefazolin (66.7%), ciprofloxacin (65%), ceftriaxone (58.3%) trimethoprim/sulfamethoxazole (53.3%), meropenem (46.7%), piperacillin/tazobactam (43.3%), tobramycin(40%), levofloxacin (31.7%), cefepime (30%), and gentamicin (30%). The pattern of antibiotics resistance in *A baumannii* isolated in our study is comparable to that reported earlier from other studies in Egypt. Yet, *A. baumannii* constituted a minor part of the examined isolates in the city of Ismailia, but all of them were MDR, 80% of them were XDR, and 60% of them were carbapenemase producers. All of the isolates were resistant to the following antibiotics: ampicillin/ sulbactam, piperacillin/tazobactam, piperacillin, trimethoprim/sulfamethoxazole, imipenem, amikacin, ceftazidime, ciprofloxacin, cefepime, cefotaxime, and ceftriaxone[28].

The use of ceftazidime, quinolone, and impinem as empirical antibiotics treatment for *A.baumannii* was recommended by Prashanth and Badrinath[29]. Nevertheless, the isolated *A. baumannii* in an Egyptian study by Zaki et al.[30] had substantial antibiotic resistance, including resistance to ampicillin/sulbactam (100%), ampicillin (100%), imipenem (100%), followed by ceftazidime (99.3%), and cefipime (96.4%). In 2020, another study was conducted by Hamady and Marei [31] in Suez Canal University Hospital and it revealed that the highest percentage of antibiotic resistance among the isolated *A. baumannii* strains was to imipenem (85.1 %). While, 80.9 % of the isolates were resistant to ceftazidime, cefotaxime and meropenem . Out of the forty- seven tested *A. baumannii*, they reported 35 isolates (74.5 %) as MDR.

Kumari et al. [32] had reported an identified resistance to cefepime (74.4%) cefotaxime (73.8%) and ceftazidime (72.5%), followed by ceftriaxone (65%), and piperacillin 65%, furthermore, 44.7% of *A. baumannii* were resistant to amikacin and 50.0% to ciprofoxacin. Other researchers reported (61%) resistance to gentamicin, (95%) cefepime, (88%) ciprofloxacin and (76%) resistant to imipenem[19]. Our results are also supported by the study of Maryam and her colleagues[33], who stated the existence of 90% resistance to sulfamethoxazole, 92% imipenem, 89% tetracycline and 84% gentamicin among the isolated *A.baumannii*.

In our study, out of the sixty isolates of *A. baumannii*, forty-five isolates (75%) were biofilm formers as detected by the tube method. MDR isolates represented (97.8%) of the biofilm producing *A. baumannii* and a significant relationship was noticed between the multidrug resistance and the ability to form biofilm.

A study in Taiwan had used crystal violet staining to identify the biofilm formation ability of 154 *A. baumannii* isolates and they further reported 45.4%, 32.5%, and 15.6% as strong, medium and weak biofilm-forming isolates respectively. Getting along with our results, the authors revealed that the MDR isolates have a significantly higher ability of biofilm formation[34]. In another study, 84 % of *A. baumannii* recovered from patients with nosocomial infections were biofilm forming pathogens and about 95% of the biofilm formers were MDR [19].

Our results are consistent with Abdi-Ali et al. [5] who utilized the microtiter plate and the test tube methods (under static conditions) to study the biofilm formation among 75 *A. baumannii* isolates and reported 75 % and 77% biofilm formers respectively. A significant

relationship between the power of biofilm formation and antibiotic resistance was also found; considering that, more than 90% of biofilm forming bacteria were MDR. Similar results were recorded by Hassan et al. [13] who observed a higher antibiotic resistance in biofilm producing bacteria than non-biofilm producers, which was in line with the findings of other studies [35,36] .

The frequency of biofilm-related genes among *A. baumannii* isolates as detected by PCR revealed that *csu E* gene was identified in 58 isolates (96.7%). However, *aba I, bap, omp A* , and *bla* $_{PER-1}$genes were identified in 53 isolates (88.3%) , 29 isolates (68.3%), 41 isolates (48.3%) and 24 isolates (40%) respectively . Our results are parallel to Yang et al.[34] with regard to *bla* $_{PER-1}$ gene distribution (about 38%). Contrariwise, the frequency of *bap, ompA, and csuE* genes were not consistent with our results.

In consistency with our results, a high frequency (100% and 93.8%) of *csuE* in *A. baumannii* isolates, was reported in two previous studies by Ghasemi et al.[7] and Sung, [37] respectively. Additional studies indicated that the frequencies of the *csuE* and *bap* genes were respectively, 93.2% and 70.3% [21].

A study performed in Iraq by Saadulla and Muhammed[24] had recorded a ratio of (73.58%) for *csuE* gene, and (79.24%) for *bap* gene. On the other hand, bla_{PER-1} was less common (40%) among the *A. baumannii* strains isolated in our study; a result near to reports from two studies belong to Qi et al.[38] and Vali et al. [39] that found bla_{PER-1} in 30.2% and 18% of isolated *A. baumannii* respectively .

The findings of our research with regard to the association between the studied genes and biofilm formation revealed that (*csu E , bap, aba I and bla* $_{PER-1}$) genes have a statistically significant relation to the development of biofilm ($P<0.05$). While (*omp A*) gene was insignificantly related to biofilm production ($P>0.05$) .In agreement, Yang et al. [34] advocated the notion that biofilm forming ability of *A. baumannii* is linked to *csuE, bap,* and bla_{PER-1}, genes.

Some studies reported a significant association between the frequency of bla_{PER-1},*bap* and biofilm formation in all *A. baumannii* isolates however, the relationship between the *csuE* gene and biofilm formation was found to be insignificant [21] .

CONCLUSION

Biofilm formation by *A. baumannii* is significantly related to multidrug resistance, with high frequency of biofilm-related genes. Several genes are involved in biofilm formation, the most commonly detected ones are *csu E, and bap.* A significant relationship is recognized between *csu E, bap, aba I, bla* $_{PER-1}$ and biofilm formation.

Higher rates of MDR and XDR in *A. baumannii* clinical isolates with great impact on hospital stay, morbidity and mortality is giving alarm to the infection control strategies and the increased need to strengthen surveillance and monitoring of antimicrobial resistance among *A. baumannii* in hospitals environment.

The article had not been published or under consideration by another journal or any other reviewed media. No financial or non-financial conflict of interest have been declared by authors. All authors participated equally to the manuscript and approved the version submitted.

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