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ORIGINAL ARTICLE

Virulence Molecular Epidemiology of Clinical Carbapenem-Resistant *Acinetobacter baumannii*: A Report from Jordan

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Abstract

Background and Aims: Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is an opportunistic Gram-negative pathogen that plays a significant role in healthcare-associated infections, leading to severe health complications. While numerous studies have focused on the antibiotic-resistance epidemiology of *A. baumannii*, fewer have investigated its molecular virulence epidemiology. This study aimed to explore CRAB virulence genes, their biofilm-forming capabilities, and the correlation between biofilm formation and both biofilm-associated virulence genes and carbapenemase-encoding resistance genes.

Materials and Methods: A total of 110 CRAB clinical isolates were collected from two hospitals in Jordan between 2018 and 2019. These isolates were screened for at least seven virulence genes using polymerase chain reaction, and biofilm formation ability was evaluated using the microtiter plate method.

Results: the prevalence of the *bap, OmpA, surA, PLD, paaE, basD,* and *traT* virulence genes was 99.1%, 98.2%, 98.2%, 95.5%, 89.1%, 86.4%, and 8.2%, respectively. Overall, 86.4% of the isolates demonstrated biofilm-forming ability, classified as weak (28.2%), moderate (36.4%) and strong (21.8%). No statistically significant correlation was observed between biofilm production and the presence of *bap, OmpA*, or the carbapenemase-encoding gene (*VIM* gene). However, a significant relationship was identified between the carbapenemase-encoding gene (*OXA-23* gene) and biofilm production.

Conclusions: CRAB infections pose a substantial threat in healthcare settings. This study underscores the critical need to enhance infection control measures in healthcare facilities to prevent CRAB outbreaks. To our knowledge, this is the first study in Jordan to examine the prevalence of virulence genes among clinical CRAB isolates.

Keywords: Acinetobacter, carbapenem, virulence, PCR, biofilm.

1. INTRODUCTION

Acinetobacter baumannii is a ubiquitous bacterium commonly found in soil, water, animals, and humans. It is considered part of the normal flora of the skin, mucosal membranes, and the respiratory tract [1]. In addition, this opportunistic pathogen is prevalent in hospital environments and is recognized as a major cause of healthcare-associated infections. *A. baumannii* is a Gram-negative, non-lactose fermenter, aerobic, non-motile bacterium that is oxidase-negative, indole-negative, citrate-positive, and catalase-positive [2].

The ability to form biofilm, along with its resistance to a wide range of antibiotics and other virulence factors, enables *A. baumannii* to withstand desiccation and disinfectants, allowing it to colonize both biotic and abiotic surfaces. This contributes to its significance as a nosocomial pathogen [3]. *A. baumannii* can contaminate medical equipment, such as ventilators, arterial pressure monitors, humidifiers, washbasins, respirometers and dialysis machines, which serve as reservoirs for hospital-acquired pneumonia, urinary tract infections, bacteremia, wound infection and meningitis [1].

The pathogenesis of *A. baumannii* involves a wide range of virulence factors, including outer membrane proteins, lipopolysaccharide, capsule, phospholipase, nutrient-acquisition systems, efflux pumps, protein secretion systems, quorum sensing, and biofilm formation. These factors play essential roles in facilitating bacterial survival, colonization, and infection [4,5].

A. baumannii, initially susceptible to antibiotics, developed significant resistance in the 1980s-1990s [6]. Carbapenem-resistant *A. baumannii* (CRAB) is linked to high morbidity, mortality, and global nosocomial outbreaks [6]. Resistance primarily arises from beta-lactamase enzymes, including *OXA-23, OXA-51, OXA-53, IMP* and *VIM*. Limited treatment options make CRAB infections challenging to manage [7].

A. baumannii is classified as a member of the ESKAPE group of pathogens, which comprises six antibiotic-resistant bacteria commonly associated with hospital-acquired infections; *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter species* [8]. The World Health Organization (WHO) has designated CRAB as a critical priority pathogen, emphasizing the urgent need for further research and new drug development [3].

CRAB is rapidly spreading globally, increasing morbidity and mortality in hospitals, particularly in Mediterranean countries with multidrug resistance [9]. While in Jordan, studies focused on CRAB clinical epidemiology and antibiotic resistance patterns, virulence epidemiology remains unexplored. This study assessed the prevalence of virulence genes and biofilm formation in CRAB to better understand its pathogenic potential and improve infection control.

2. METHODOLOGY

2.1 Sample Collection

In this study, a total of 110 clinical isolates of carbapenemase-producing *A. baumannii* were obtained from the Faculty of Medicine at Hashemite University. These isolates had been previously collected from Prince Hamzah Hospital and the Islamic Hospital between January 2018 and December 2019. They were preserved at -70 °C in deep freeze storage.

2.2 Demographic Data Recruitment

Patient data were retrieved from the archive department using patient identification codes.

2.3 Isolate Characteristics

The clinical isolates were previously published by Al-Tamimi et al [9]. They were obtained from various sample sources, including sputum, wounds, blood, urine, cerebrospinal fluid and bronchial lavage. The majority of samples were collected from the intensive care unit (ICU). The isolates were identified using standard bacteriological methods and confirmed by polymerase chain reaction (PCR). They exhibited high resistance to a broad spectrum of antibiotics, except for colistin.

2.4 Bacterial Reactivation

All CRAB isolates were reactivated by culturing the preserved cells in nutrient broth media (Himedia, India) and incubating them at 37 °C for 18-24 hours. The resulting bacterial suspension was centrifuged at 5000 *xg* for 10 minutes, after which the supernatant was discarded. The bacterial pellet was sub-cultured on MacConkey agar and incubated overnight. On the next day, CRAB colonies were collected and introduced for biofilm formation assay and DNA extraction.

2.5 Detection of Biofilm Production

The biofilm production test was performed using the microtiter plate (MTP) method, as previously described [4], with minor modifications. Bacterial colonies were transferred into 10 mL of tryptic soya broth (TSB), and the turbidity of the suspension was adjusted to a final concentration of 10^6 CFU/mL using a turbidimeter. Aliquots of 200 µL fresh bacterial suspension were then dispensed into a 96-well flat-bottom polystyrene microtiter plate and incubated at 37 °C for 24 hours. Following incubation, the wells' contents were discarded and washed three times with 200 µL of sterile phosphate-buffered saline (PBS). To fix adherent bacteria, 200 µL of 99% methanol was added for 15 minutes. The wells were then emptied and air-dried. The plates were subsequently stained with 1% Hucker crystal violet for 20 minutes. Excess stain was removed by rinsing with running tap water.

The plates were air-dried, and 160 µL of 33% (v/v) glacial acetic acid was added to each well to resolubilize the dye bound to the adhered bacterial cells. The mean optical density (OD) at 570 nm of the non-biofilm producing *A. baumannii* was used as the OD cut-off value (ODc), measured with an automated Biotic Synergy HTX Multi-Mode Reader [10]. The quantitative biofilm assay was performed in triplicate for all isolates, with sterile TSB used as the negative control. The OD results for all tests were categorized into four groups: (1) OD ≤ ODc: non-biofilm producer; (2) ODc < OD ≤ 2× ODc: weak biofilm producer; (3) 2× ODc < OD ≤ 4 × ODc : moderate biofilm producer; and (4) 4× ODc < OD: strong biofilm producer [4].

2.6 Polymerase Chain Reaction for Seven Virulence Genes

2.6.1 DNA extraction

DNA was extracted from CRAB broth preparations using the commercial extraction DNeasy tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The final DNA yield (minimum 50 ng/µL per sample) was used for PCR analysis.

2.6.2 Primers

Primers for the seven virulence genes (*bap, surA1*, *basD, PLD, OmpA, paaE,* and *traT*) were previously published (table 1). These primers were commercially ordered from Macrogen Inc. (Rockville, MD), supplied in lyophilized form, and diluted with nuclease-free water to a final working concentration of 10 pmol/µL, as per the manufacturer's guidelines.

Table 1. Primers details for all seven virulence genes.

Virulence factor	Gene	Sequence	Annealing temp. (ºC)	Ref.
biofilm-associated protein	*Bap*	**F**:AGTTAAAGAAGGGCAAGAAG **R**:GGAGCACCACCTAACTGA	50	[7]
Surface antigen protein	*surA1*	**F**:CAATTGGTAGCTGGCGATCA **R**:TTAGGCGGGACTCAGCTTTT	55	[7]
Iron acquisition system	*BasD*	**F**: CTCTTGCATGGCAACACCAC **R**:CCAACGAGACCGCTTATGGT	65	[7]
Phospholipase D	*Pld*	**F**:CGTCAATTACGCCAAGCTG **R**:CTGACGCTACCTGACGGTTT	64.7	[7]
Outer membrane protein A	*OmpA*	**F**:CGCTTCTGCTGGTGCTGAAT **R**:CGTGCAGTAGCGTTAGGGTA	50	[4]
Phenylalanine catabolic pathway	*PaaE*	**F**:CTATTTAGGCGTTGCTGCGG **R**:CCTTACAACGACAGGTCGCA	64.5	[7]
Surface-exposed lipoprotein	*traT*	**F**:GGTGTGGTGCGATGAGCACAG **R**:CACGGTTCAGCCATCCCTGAG	67.9	[7]

2.6.3 PCR assay

The optimized PCR mixture for each gene contained 2.0 µL of extracted DNA in a total reaction volume of 25 µL. The mixture included 12.5 µL of 2x OneTaq quick-load PCR mix (New England Biolabs/USA), containing a reaction buffer with 5 mM dNTPs, 15 mM $MgCl_2$, stabilizers, enhancers, and DNA polymerase. Additionally, 1.0 µL of 1.0 µM of forward primer, 1.0 µL of 1.0 µM of reverse primer, and 8.5 µL nuclease-free PCR water (Avantor/USA) were added. Amplification was carried out using a PCR thermocycler (Qiagen, Germany) with standard settings. The optimized thermal profile included initial denaturation at 94 ºC for 5 minutes, followed by 30 cycles of denaturation step at 94 ºC for 45 seconds, annealing step for 15 seconds at different temperatures according to every gene (table 1) and extension step at 72 ºC for 1 minute followed by final extension step at 72 ºC for 10 minutes. A no-template negative control (PCR water) and positive controls were included in each run to monitor for amplification failure or potential contamination.

All targeted virulence genes in this study were amplified using primer set, then purified and sequenced via Sanger sequencing. The raw sequences were trimmed using BioEdit software, aligned using BLASTn on PubMed, and confirmed for gene identity, data not shown.

2.6.4 Agarose gel electrophoresis

PCR products were analyzed on a 1.5% agarose gel with ethidium bromide stain, using a 100-bp DNA ladder. Amplicons were visualized with a UV gel documentation system.

2.7 Statistical Analysis

Statistical analyses were performed using SPSS v25, with correlations assessed via the Pearson Chi-Square test. A $p\ value \leq 0.05$ was considered significant.

3. RESULTS

3.1 Demographic Data

This study investigated CRAB isolates from two major hospitals in Jordan, involving 110 patients (64% males, average age of 59.7 years). Most isolates (57%) were from patients over 60 years, with 91% from ICU. Sputum samples comprised the majority (69%) of collected specimens, figure 1.

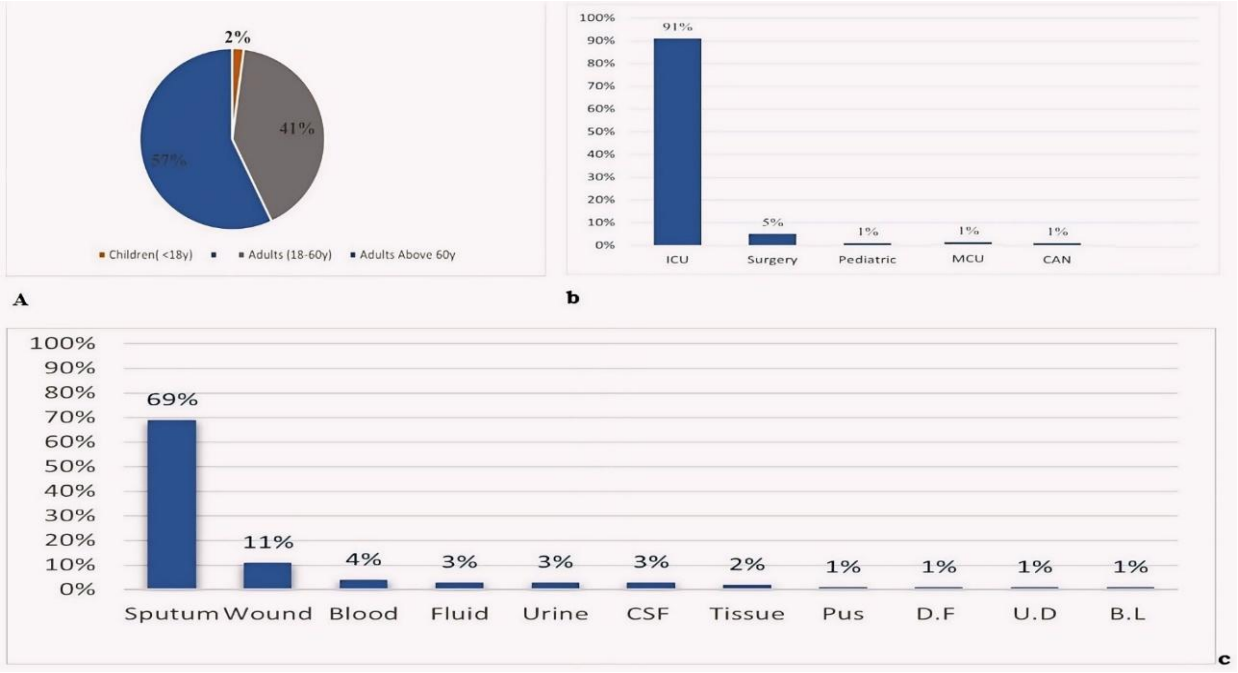

Figure1. Distribution of A: age interval, B: department source and C: sample source among 110 CRAB isolates.

CAN: central admission nursery, MCU: medical care unit, D.F: diabetic foot, U.D: urethral discharge, B.L: bronchial lavage

3.2 Distribution of Virulence Genes among CRAB Isolates

In this study, the frequencies of the examined virulence genes in CRAB isolates ranged from 8.2% for traT gene to 99.1% for bap gene, figure 2.

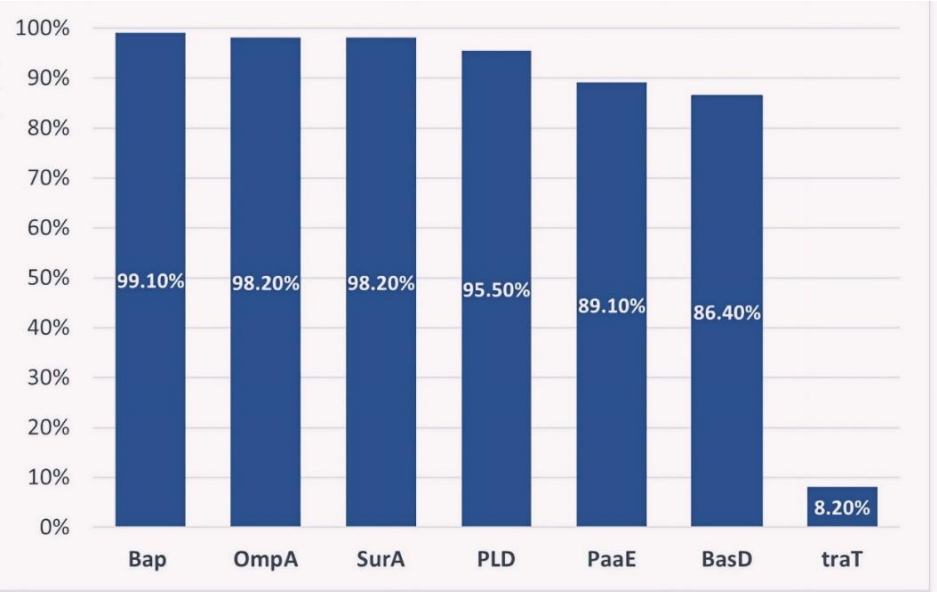

Figure 2. Percentages of the virulence genes in *A.baumannii* clinical isolates.

3.3 Biofilm Detection by Microtiter Plate Method (MTP) in CRAB

Overall, 95 out of 110 CRAB isolates (86.4%) were biofilm producers, exhibiting varying strength patterns: strong (21.8%), moderate (36.4%) and weak (28.2%), figure 3.

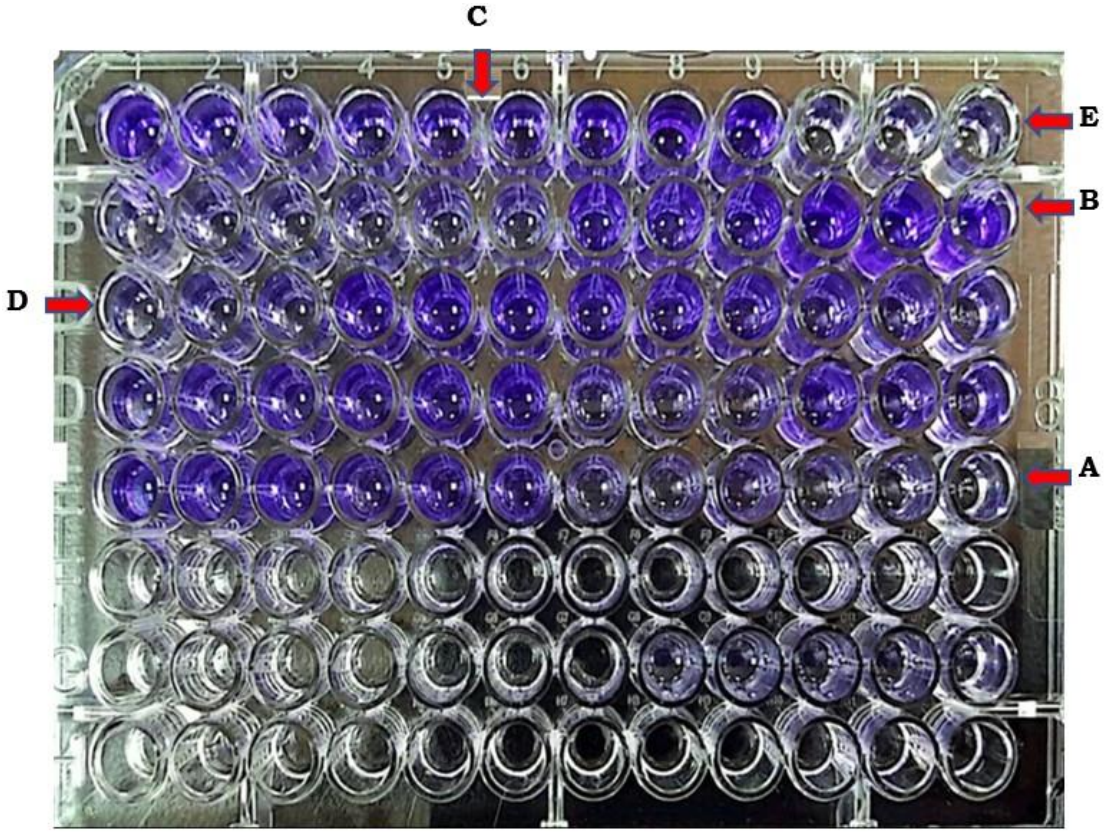

Figure 3. Microtiter plate method for detection of biofilm production. A: negative control, B: strong producer, C: moderate producer, D: weak producer, E: non producer.

3.4 Statistical Associations

Analysis of biofilm production revealed a high prevalence of *Bap* (98.9%) and *OmpA* (97.9%) among biofilm producers, though without significant associations. The *VIM* gene was found in 28.4% of biofilm producers with no positive association. However, the *OXA*-23 gene was present in all biofilm producers, with a positive association (*p value* <0.05).

4. DISCUSSION

A. baumannii, an opportunistic pathogen resilient in hospital environments, poses a global health threat due to its increasing resistance to carbapenems. This results in limited treatment options, higher morbidity, and mortality, particularly in ICU settings [11]. Research in Jordan has focused on resistance mechanisms, with limited attention to virulence factors [4]. This study investigated CRAB virulence in clinical samples from two hospitals using molecular and conventional methods.

The study involved 110 CRAB isolates, with the majority of samples (91%) collected from the ICU, which was consistent with previous literature [12,13]. The high prevalence of CRAB in the ICU may be attributed to increased exposure to various

risk factors, such as immunocompromisation, using catheters, mechanical ventilators, ventriculoperitoneal shunts, and central lines, in addition to the widespread use of antibiotics with varying activity spectra [14].

Pneumonia and other pulmonary infections are common in ICU patients, who often require mechanical ventilation and other respiratory procedures, increasing the risk of infection [15]. Accordingly, more than two-thirds (69%) of the isolates in this study were collected from respiratory samples, consistent with findings from previous studies [16,17].

The proportion of CRAB infection was higher in males than in females, which may be linked to lifestyle factors, behaviors, and a higher likelihood of males developing chronic obstructive pulmonary diseases (COPD), a condition that increases the risk of CRAB infection [18]. In terms of age, children under 18 years have a relatively low risk of infection, accounting for only 2%, likely due to less exposure to infection-predisposing factors compared to adults [19]. Similar CRAB frequencies have been reported in both genders and age groups in previous studies [20].

In this study, all virulence genes were found at high frequencies, ranging from 86.4% to 99.1%, except for *traT* gene (8.2%). These results align with or exceed those reported in other studies, which may vary based on endemicity and regional prevalence [21], table 2.

Table 2. A summary for the prevalence of CRAB virulence genes.

Virulence gene	Frequency in Jordan (this study)	Study country	Frequency range	Ref
Bap	99.1%	S. Korea, Iran Serbia, Thailand	48-100%	[4,10,22,23]
OmpA	98.2%	Iran, S. Korea,	77.1-100%	[21,24]
surA	98.2%	Iran, China	95-98%	[7,24]
PLD	95.5%	China, Poland	87.5-99%	[7,25]
PaaE	89.1%	China	88.6%	[7]
basD	86.4%	China, Poland	92-95%	[7,25]
traT	8.2%	China, Iran	0-80%	[7,23]

In the current study, the high frequency of *Bap* and *OmpA* genes was found to predict bacterial biofilm formation, indicating prolonged strain persistence in hospitals and resistance to a wide range of antibiotics [21].

Biofilm formation among CRAB isolates was evaluated in vitro using the microtiter plate assay. Biofilm formation was observed in 86.4% of these isolates, supporting the widely recognized fact that *A. baumannii* is a significant biofilm producer. Biofilms enable the bacterium to survive in harsh environments and resist antibiotic treatments [10]. This high prevalence of biofilm formation was consistent with findings from other studies conducted worldwide [10].

Out of 110 tested CRAB isolates, 86.4% were identified as biofilm producers. Among these, 31 were classified as weak biofilm producers, 40 as moderate, and 24 were strong biofilm producers. Similar results have been reported in previous studies [26],

though some discrepancies exist, with other research reporting varying percentages [27]. These differences may be attributed to the widespread presence of biofilm-associated genes among *A. baumannii* strains, variations in biofilm assay methodologies, or differing environmental conditions [28].

For example, Abdi-Ali et al. [29] applied both test tube and microtiter plate methods to evaluate biofilm formation. Using the microtiter plate method, they reported 25% non-biofilm producers, 41% weak, 10% moderate, and 18% strong biofilm producers. In contrast, the test tube method identified 18% non-producers, 42% weak, 18% moderate, and 22% strong biofilm producers.

It is crucial to note that in vitro methods for assessing biofilm formation may not accurately reflect the complexity of biofilm development in clinical settings [28]. Notably, all CRAB isolates in this study were obtained from clinical samples. Previous research has shown that clinical CRAB isolates tend to exhibit a stronger capacity for strong biofilm formation compared to environmental isolates [30].

This study found high prevalence of biofilm-associated genes bap (97.9%) and *OmpA* (98.9%) in CRAB isolates but no significant correlation with biofilm formation. This suggest biofilm development is influenced by complex genetic and environmental factors [4], warranting further research

Additionally, the relationship between biofilm formation and carbapenem resistance genes (*VIM* and *OXA-23*) was analyzed. While no significant correlation was found with *VIM*, a significant correlation was observed with *OXA-23* (p *value*< 0.05), consistent with findings from an earlier study conducted in Turkey [31]. However, the associations between biofilm formation and antibiotic resistance remain controversial [32]. The specific resistance determinants present in *A. baumannii* may influence its biofilm-forming ability. Moreover, biofilm formation is generally more strongly associated with CRAB strains the with antibiotic susceptible strains [32].

5. CONCLUSION

This study highlighted the increased prevalence of six virulence genes (excluding *traT*) among CRAB isolates, indicating a strong virulence potential. These genes enhance the adaptability and survival of CRAB in diverse environments. The observed high capacity for biofilm production further contributes to the persistence of these strains in hospital settings and their resistance to treatment, posing significant challenges for infection control and clinical management.

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Conflicts of Interest

The authors declare no conflict of interest

Author's contributions

Conceptualization, A.M.Z.; Data curation, D.A.B. and H.A.B; Formal analysis, D.A.B. and H.A.B; Funding acquisition, A.M.Z and R.B.S; Investigation, D.A.B.; Methodology, D.A.B. and H.A.B; Project administration, A.M.Z.; Resources, M.A.T.; Software, D.A.B. and H.A.M; Supervision, A.M.Z.; Validation, H.M.S, R.B.S, D.S.H., H.A.M and T.M.; Visualization, H.M.S and R.B.S;

Writing – original draft, A.M.Z.; Writing – review & editing, A.M.Z.; H.M.S and R.B.S.

Ethical approval

This study was approved by the Institutional Review Board (IRB) at Hashemite University under the approval number (No.16/3/2022/2023).

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الوبائية الجزيئية للضراوة في عزلات *Acinetobacter baumannii* المقاومة للكاربابينيم: تقرير من الأردن

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الملخص

الخلفية والأهداف: تُعدّ بكتيريا *Acinetobacter baumannii* المقاومة للكاربابينيم (CRAB) مُمْرِضًا انتهازيًا سلبي الغرام، يلعب دورًا هامًا في العدوى المرتبطة بالرعاية الصحية، مما يؤدي إلى مضاعفات صحية خطيرة. في حين ركزت دراسات عديدة على وبائيات مقاومة المضادات الحيوية لبكتيريا *Acinetobacter baumannii*، إلا أن دراسات قليلة تناولت وبائيات ضراوتها الجزيئية. هدفت هذه الدراسة إلى استكشاف جينات ضراوة CRAB، وقدراتها على تكوين الأغشية الحيوية، والعلاقة بين تكوين الأغشية الحيوية وجينات الضراوة المرتبطة بها وجينات المقاومة المشفرة للكاربابينيماز.

المواد والطرق: جُمعت 110 عزلات سريرية لبكتيريا CRAB من مستشفيين في الأردن بين عامي 2018 و2019. فُحصت هذه العزلات بحثًا عن سبعة جينات ضراوة على الأقل باستخدام تفاعل البوليميراز المتسلسل، وقُيّمت قدرتها على تكوين الأغشية الحيوية باستخدام طريقة صفيحة الميكروتيتر.

النتائج: بلغ معدل انتشار جينات الضراوة bap، وOmpA، وsurA، وPLD، وpaaE، وbasD، و traT 99.1%، و98.2%، و98.2%، و95.5%، و89.1%، و86.4%، و8.2% على التوالي. وبشكل عام، أظهرت 86.4% من العزلات قدرة على تكوين الأغشية الحيوية، مصنفة على أنها ضعيفة (28.2%)، ومتوسطة (36.4%)، وقوية (21.8%). ولم يُلاحظ أي ارتباط ذي دلالة إحصائية بين إنتاج الأغشية الحيوية ووجود bap، أو OmpA، أو جين ترميز الكاربابينيماز (VIM). ومع ذلك، وُجدت علاقة ذات دلالة إحصائية بين جين ترميز الكاربابينيماز (OXA-23) وإنتاج الأغشية الحيوية.

الاستنتاجات: تُشكل عدوى CRAB تهديدًا كبيرًا في مرافق الرعاية الصحية. تُبرز هذه الدراسة الحاجة المُلحة لتعزيز إجراءات مكافحة العدوى في مرافق الرعاية الصحية للوقاية من تفشي عدوى CRAB. وعلى حد علمنا، تُعدّ هذه الدراسة الأولى في الأردن التي تبحث في مدى انتشار جينات الضراوة بين عزلات CRAB السريرية.

الكلمات الدالة: الوبائية الجزيئية، تفاعل البوليميراز المتسلسل، الكاربابينيم، الضراوه، الاغشيه الحيويه.