Iraqi Journal of Biotechnology, 2024, Vol. 23, No. 3, 146-154

Molecular Detection of some Efflux Pump Genes among *Acinetobacter baumannii* Isolated from Intensive Care Units

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Received: October 22, 2023 / Accepted: February 14, 2024 / Published: December 30, 2024

Abstract: *Acinetobacter baumannii* is a bacterium of considerable importance that is frequently linked to nosocomial infections, notably within the confines of intensive care units (ICUs). This specific bacteria , known for its opportunistic behaviour, can be easily acquired from many sources like water, soil, and healthcare establishments. The aim of this study was to determine the prevalence and patterns of resistance exhibited by *Acinetobacter baumannii* strains that were isolated from the intensive care units (ICUs) of Diwaniyah Hospitals. Over a span of six months, specifically from January to June 2023, a total of 100 specimens were gathered from the Intensive Care Units (ICUs) of hospitals in Diwaniyah. The isolates were identified as *A. baumannii* through the use of traditional phenotypic and biochemical testing, and this identification was further validated by conducting PCR assays targeting the *16sRNA* and *blaOXA-51*like gene. The susceptibility of isolates was assessed using the standard disk diffusion method. From total of 100 sputum sample, 20 isolates were identified as *A. baumannii*. The antimicrobial susceptibility patterns of isolates showed that 100 % of isolates were resistant to Azithromycin and ceftriaxone, 85% to Levofloxacin, 100% to imipenem meropenem. The results of the study demonstrated that the presence of *adeA, adeB, and abeR* genes in *A. baumannii* isolates was seen in all isolates The findings of this investigation demonstrated a notable level of resistance exhibited by *A. baumannii* towards a diverse array of antimicrobial agents.

Keywords: *Acinetobacter baumannii*, Antimicrobial Susceptibility Test ,Efflux pumps, Carbapenem resistance.

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Introduction

Acinetobacter baumannii is classified as a Gram-negative coccobacilli non-glucose fermentative opportunistic pathogen (1). *Acinetobacter baumannii* is considered to be a significant nosocomial pathogen because to its prolonged survival inside hospital settings, its resistance to various antimicrobial agents, and its potential to colonize susceptible individuals who are undergoing treatment with broad-spectrum antibiotics (2). many studies have established a correlation between this phenomenon and the occurrence of infections in many anatomical sites, such as the urinary tract, skin and soft tissue, pneumonia, and bloodstream infections, particularly among those with compromised immune systems (3-4). The global observation of

multidrug-resistant *Acinetobacter baumannii* (MDR *A. baumannii*): denoting a strain of *A. baumannii* that exhibits resistance to a minimum of three distinct antibiotic groups, namely penicillins and cephalosporins (including inhibitor combinations): fluoroquinolones, and aminoglycosides, has been documented. The aforementioned occurrence has been correlated with a substantial rise in morbidity, death, and expenses related to treatment (5).

Acinetobacter spp. display multidrug resistance through generation of β-lactamases, changes in external layer proteins (OMPs) and penicillin-binding proteins (PBPs): and expanded movement of efflux pumps (7).

Previously, *A. baumannii* was regarded as a pathogen of low significance. However, it has since emerged as a prominent causative agent, responsible for around 20% of infections occurring in intensive care units (8,9).

Acinetobacter baumannii exhibits resistance to factors such as dehydration, ultraviolet (UV) radiation, commonly used chemical disinfectants, and detergents.The organism has the ability to persist in arid environmental circumstances and is found in several areas within the hospital setting, including shaded areas, beds, furniture, and clinical equipment. Consequently, the primary mechanism of transmission is through the hands of healthcare personnel, originating from several sources. In times, *Acinetobacter baumannii* has become increasingly prevalent as both an endemic and epidemic pathogen inside hospital settings (12). Within the clinical setting, *Acinetobacter baumannii* poses a significant risk due to its ability to colonize and infect highly immunocompromised patients in intensive care units (ICUs) (13,14,15). Limited options are available for eradicating *A. baumannii* from commonly used medical equipment, notably catheter-related devices, in hospital settings (16).

Material and method

Samples collection

One hundred Sputum specimens were collected from patient admitted to ICUs of Diwaniyah hospital between January 2023, and June 2023.

Isolation and Identification

The specimens were subjected to inoculation on MacConkey agar medium and *A. baumannii* Chrom agar. Subsequently, they were cultured for a duration of 24 hours at a temperature of 37 C˚ . The identification of culture growth as *A. baumannii* growth was achieved through the observation of colony morphology, and by the microscopic examination (17).

Antimicrobial Susceptibility Test for *Acinetobacter* spp

The isolates were subjected to Kirby-Bauer disc diffusion sensitivity testing, following the guidelines established by the Clinical and Laboratory Standard Institute (CLSI): using Mueller-Hinton Agar(18). The reference provided is in the format of a citation, indicating that the user is referring to The following antimicrobial agents were employed in the study: meropenem (10 µg): imipenem (10 µg): azithromycin (15 µg): ciprofloxacin (30 µg): and levofloxacin (5 µg). Acinetobacter has reduced resistance to the antimicrobial disc of meropenem (10 µg): as indicated by an inhibition zone diameter of < 11 mm.

EtBr-agar cartwheel method

The phenotypic identification of antibiotic resistance caused by efflux pumps in *Acinetobacter baumannii* carbapenem resistant isolates was

conducted using the approach described in reference (19).

The strains were cultivated in 5 ml of the suitable liquid medium until they attained an optical density (OD) of 0.6 at a wavelength of 600 nm. The optical density (OD) of the cultures was adjusted using phosphate-buffered saline (PBS) to a value of 0.5 based on the McFarland standard. Agar plates were made with varying concentrations of ethidium bromide (EtBr) ranging from 0 to 2.5 mg/l. The plates were subsequently partitioned into up to four sectors through the use of radial lines, forming a cartwheel pattern. The cultures were inoculated into Ethidium Bromide (EtBr)-agar plates, commencing from the central region of the plate and extending outwards towards the periphery. Subsequently, the EtBr-agar plates were subjected to incubation at a temperature of 37˚C for a duration of 16 hours, followed by examination under an appropriate UV light source.

Molecular identification

The molecular identification of *A. baumannii* isolates was determined by performing Polymerase Chain Reaction (PCR) utilizing the *16s rRNA* and *blaOXA-51* gene as specific gene. The genomic DNA extraction of bacterial isolates was conducted in the initial phase using a commercially available genomic DNA purification kit. The instructions provided by the manufacturer (Promega, USA) were followed, with minor adjustments. Following that, the DNA obtained from the *A. baumannii* isolates was evaluated using spectrophotometric analysis, specifically the nano drop devise. The primers utilized for the identification of *A. baumannii* using PCR, specifically targeting these genes, found in (Table 1).

Table (1): The primer sequence for *A. baumannii* of OXA51-PCR and 16SrRNA-PCR and there product size

Primer	Sequence 5'-3'	PCR product (bp)	NCBI Reference code
***blaOXA-51* -F**	TAATGCTTTGATCGGCCTTG	353	(33)
***blaOXA-51*-R**	TGGATTGCACTTCATCTTGG		
***16S rRNA* -F**	CACCAGTGGGGAATGAGACC	571	NZ_CP043953.1
***16S rRNA* -R**	GTTAGCTGCGCCACTAAAGC		

And for the efflux pump genes detection three genes were used AdeA, AdeB and AdeR and the primers used for detecting these genes as in (Table 2).

Table (2): The primer sequence for *A. baumannii* of OXA51-PCR and 16SrRNA-PCR and there product size

Primer	Sequence 5'-3'	PCR product (bp)	NCBI Reference code
adeA -F	ATGTTGACCCGGAAACAGGC	409	NZ_CP043953.1
adeA -R	GGTTGTGCCCCTTCAGCTAT		
adeB -F	GCGAATAGTACGGAAGGCGA	554	NZ_CP043953.1
adeB -R	ATTGCTTCACCCGATGACGT		
adeR -F	TCCTGTGATCATGCTGACGG	449	NZ_CP043953.1:
adeR -R	CCACGCCACGCACATTAATT		

The PCR master mix reactions for all genes were prepared using the GoTaq ®Green PCR master kit, following the instructions provided by the manufacturer (Table 3).

Table (3): PCR conditions to detect the *abeA* , *abeB and abeR* genes of *A.baumannii* isolates

No.	Step	Temperature	Time	No. of Cycles
1	Initial denaturation	95°C	5 min.	1
2	Denaturation	95°C	30 sec.	30
3	Annealing	58°C	30 sec.	
4	Extension	72°C	60 sec.	
5	Final extension	72°C	3 min.	1
6	Storage	4°C	∞	-

Results and discussion

In this study, the cultured and isolated *A.baumannii* strains from clinical samples, ensuring the purity and viability of the strains used for subsequent experiments. The strains were confirmed to be *A. baumannii* through molecular identification, such as PCR targeting specific *A. baumannii* genes.

Acinetobacter baumannii on chrome agar medium appeared as bright salmon-red colonies as (Figure 1). In gram stain all isolates appeared as Gram-negative coccobacilli and occasionally arranged in diplococci.

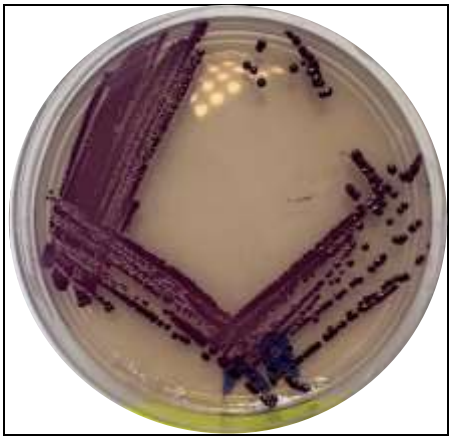

Figure (1)The morphology of *A. baumannii* colonies on *HiCrome Acinetobacter* medium after incubation at 37ºC for 24 hours

Out of a total of 100 sputum samples , a subset of 20 isolates were identified as belonging to the *A. baumannii* species using a phenotypic approach. PCR was used to confirm the identity of all the isolates. The outcomes of the polymerase chain reaction (PCR) analysis for the 16sRNA and blaOXA 51 genes were observed. As depicted in (Figure 2). The findings of antimicrobial patterns of the isolates revealed that all of the isolates (100%) demonstrated resistance to Azithromycin and ciprofloxacin, while 85% exhibited resistance to Levofloxacin. Additionally, all isolates displayed (100%) resistance to imipenem meropenem.

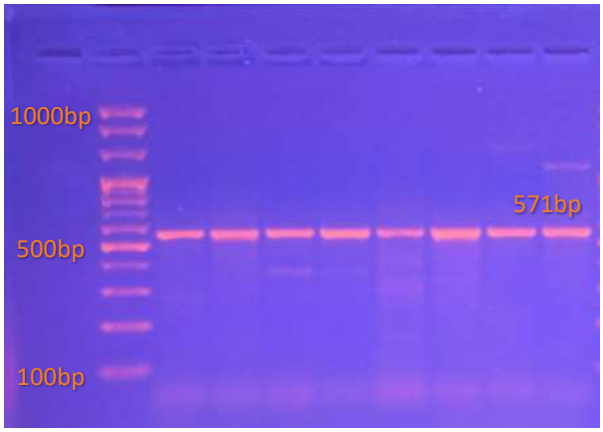

Figure (2): Agarose gel electrophoresis of PCR products for the 16SrRNA Gene.

To ensure the accuracy of our findings, we compared the results obtained using the Cartwheel method with traditional diagnostic techniques, such as PCR. The Cartwheel method consistently provided reliable results and showed excellent concordance with conventional methods, establishing its effectiveness as a diagnostic tool for fluxes pump gene expression in *A. baumannii*. On cart wheel method all isolate were fluoresced at a concentration of 2 mg/ml of EtBr plate as in (Figure 3) that indicate the presence of the efflux pump apparatus in our isolate and that has been confirmed by the results of PCR.

Following the identification of isolates exhibiting efflux pump activity (n=20): the prevalence of *adeA, adeB,* and *abeR* genes was assessed using polymerase chain reaction (PCR) analysis regarding these isolates. The results of the study demonstrated that the presence of *adeA, adeB, and abeR* genes in *A. baumannii* isolates was seen in all isolates, as depicted in the accompanying (Figures 4, 5, 6).

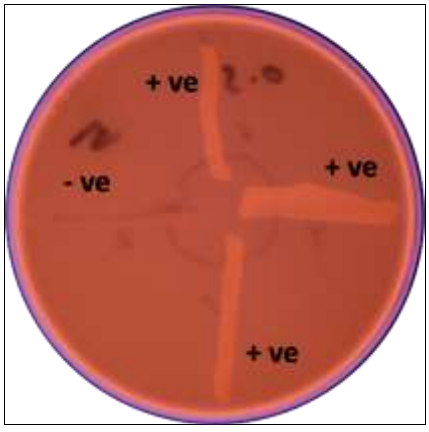

Figure (3): *A. baumannii* EtBr plate 2 mg/ml at 37ºC for 24hrs

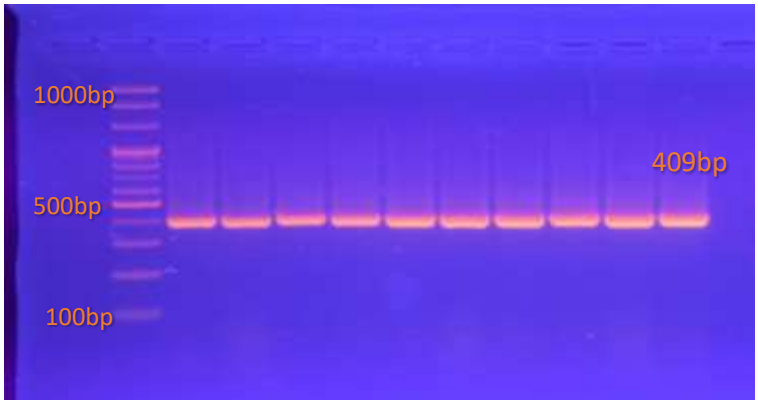

Figure (4): The PCR results for the adeA Gene were subjected to agarose gel electrophoresis.

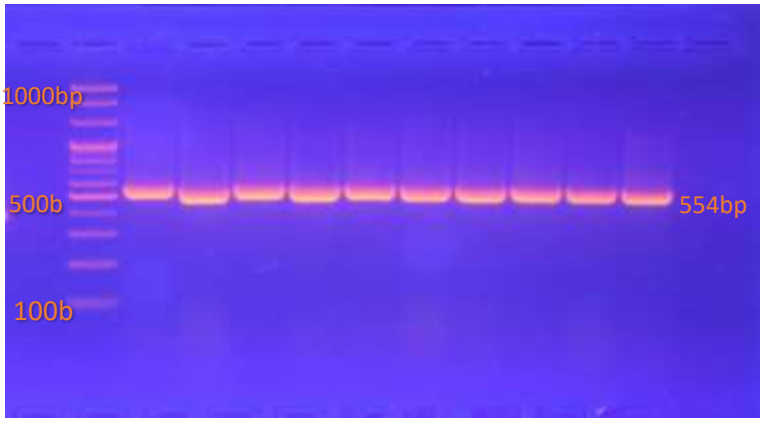

Figure (5): The PCR results for the adeB Gene were subjected to agarose gel electrophoresis.

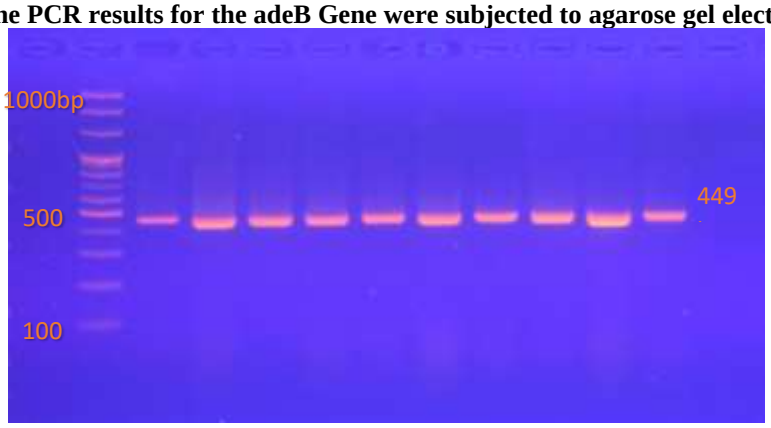

Figure (6): The PCR results for the adeR Gene were subjected to agarose gel electrophoresis.

In recent years, there has been a notable rise in nosocomial infections, which are acquired during the course of medical treatment within a hospital setting. Certain diseases are attributed to a bacterium known as *A. baumannii*, which is classified as a pathogen that exploits compromised immune systems. The prevalence of this bacterium is on the rise. The management of bacteria, particularly those exhibiting resistance to several antibiotics and possessing extensive resistance genes, is a significant concern(20). In the conducted study, a total of 100 samples were collected from the intensive care unit. Among these samples, 20 isolates (20%) were identified as *A. baumannii*.

In addition to Al-Ahmer and colleagues, the diagnostic assessment

was conducted on a total of 47 samples. The polymerase chain reaction (PCR) analysis revealed that 35 of these samples had a positive outcome, as determined by the discovery of the *bla-OXA-51*-like gene (21).

The outbreak of drug-resistant strains of *A. baumannii* in the intensive care unit (ICU) was described by Wang *et al*. (2003). It is worth mentioning that all of the strains that were found demonstrated resistance to aztreonam, amikacin, ampicillin-sulbactam, ceftazidime, cefepime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, tazobactam, and ticarcillin clavulonic acid. However, they revealed sensitivity to polymyxin B. (22).

Al-Taliby and Al-Daraghi study revealed that all 20 isolates exhibited resistance to a range of antibiotics, namely Ceftazidime, Amoxillin, Carbencillin, Ertapime, cefepime, Meropenem, cefoxitin, Azetronam, Tobramycin, Oxacillin, and Cefotaxime. The resistance rates were found to be 100%, 85%, 90%, 80%, 75%, 90%, 90%, 80%, 70%, and 80% respectively(23).

In a study conducted by Sadeghifard *et al*. (2010): it was demonstrated that every strain of *A. baumannii* exhibited complete resistance (100%) to aztreonam, cefotaxime, ceftazidime, ceftriaxone, meropenem, and ticarcillin-clavulanate.(24) In a separate investigation, the researchers demonstrated that the bacteria that were isolated exhibited complete resistance (100%) to tetracycline. Furthermore, the bacteria had a resistance rate of 95.2% to both gentamicin and amikacin, while a resistance rate of 90.5% was observed for ceftazidime (25).

Additional research conducted in Asia and the Middle East has revealed the existence of a high incidence of multidrug-resistant *Acinetobacter baumannii* in these geographical areas (26,27).

In a survey carried out in 2010 in Saudi Arabia, a total of 1210 isolates were collected from various samples, including 469 from breathing samples, 400 from blood samples, 235 from injury/tissue samples, 56 from urine samples, 35 from nasal swabs, and 15 from cerebrospinal fluid (CSF) samples obtained from patients in the intensive care unit (ICU). The survey revealed that 40.9% of the isolates were identified as *A. baumannii*, 19.4% as *Klebsiella pneumoniae*, and 16.3% as *Pseudomonas aeruginosa* (28).

In this study, the EB-agar cartwheel approach was employed to identify overexpressed efflux systems. The results indicated that efflux activity was observed in all isolates, which contrasts with the findings of a previous study (31) where efflux activity was detected in only 85.7% of the isolates.

This work employed the *adeA*, *adeB*, and *abeR* genes as molecular markers for the purpose of identifying efflux pump activity. The obtained results indicated a 100% presence of these genes across all isolates. In contrast to the findings reported in Rafiei *et.al* (2022) the researchers utilized the *adeB*, *adeG*, *adeJ*, and *abeM* genes, observing frequencies of 100%, 90%, 86%, and 98% respectively(32).

The intensive care unit (ICU) places significant emphasis on infection control due to the prolonged hospitalization of patients and the severity of their illnesses. Prior studies have demonstrated that *A. baumannii* is the predominant infection encountered in the intensive care unit (ICU) or hospital environment. The acquisition of knowledge on the specific locations where *Acinetobacter* exhibits resistance

to particular therapies would facilitate medical practitioners in determining the appropriate therapeutic approach.

Conclusion

The study revealed that a significant number of *A. baumannii* strains demonstrate resistance to an antibacterial agent. Hence, it is imperative to employ appropriate methodologies in order to effectively mitigate the dissemination of these strains within the intensive care unit (ICU).

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