Academicia Globe:
Inderscience Research

ISSN: 2776-1010 Volume 3, Issue 6, June, 2022

MOLECULAR DETECTION AND ANTIBIOTIC RESISTANCE PROFILES OF ACINETOBACTER BAUMANNI

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Abstract
Acinetobacter baumannii is a Gram-negative, coccobacillus, and biofilm-forming bacteria. Aims of the current study is detection, isolation, molecular diagnosis of A. baumanni spp that isolated from hospitalized patients with burns at Baquba Teaching hospital in Diyala province. (50) Samples are collected from patients with burn infections. The findings are included that percentage of isolates of A. baumannii was 10/50 (20%) in burns infections. The samples are cultured on MacConkey, blood, and tested by gram stain and biochemical examination. The confirmed molecular diagnosis of the isolates was made using designed primers of the 16SrRNA gene for the PCR technique. Furthermore, the results include the use of (15) antibiotics against A. baumannii and showed that all clinical isolates were resistant to many antibiotics using the Vitek-2 system. All isolates were resisted (8) tested antibiotics. Imipenem did not affect 90% of A. baumannii. 10% of the isolates were sensitive to Tobramycin, Gentamicin, and Amikacin. 50% of them could resist Trimethoprim-sulfamethoxazole. Finally, all the isolates could not resist Colistin, and 10% showed resistance against Tigecycline and Minocycline. Eight isolates 8/10 (80%), displayed gyrA gene, while 5/10 (50%) of isolates had bla VIM gene. Moreover, all A. baumannii isolates could form biofilms. A. baumannii, one of the most important pathogens, has high resistance to used antibiotics but shows high sensitivity to Colistin, and can generate strong biofilms.
Keywords: A. baumannii, 16SrRNA, Biofilm-forming, Vitek-2 system

Introduction

A. baumannii is a Gram-negative, non-lactose fermenting, coccobacillus, strictly aerobic, and its pathogenic isolates grow at 42-37°C. It is reported primarily as a healthcare-associated pathogen causing several nosocomial infections, including; septicemia, bacteremia, ventilator-associated pneumonia (VIP), and urinary tract infections (UTI) (1). Biofilms formed by bacterial cells can clarify their antimicrobial resistance and persistence on abiotic surfaces in the existence of disinfectants and/or desiccation agents (2).
The development of A. baumannii strains resistant to broad-spectrum antibacterial drugs in hospital environments has been a significant health burden due to the insufficient therapeutic opportunities for infections caused by this pathogen (1). Multidrug-resistant A. baumannii has a high ability of resistance to numerous types of antibiotic classes; Antibiotic resistance mechanisms may be divided into three classes based on their function. First and foremost, antibiotic resistance may be established by decreasing membrane fluidity over the membranes or raising the outflow of the antibiotic, so blocking access to a specific site by the antibiotic. Secondly, bacteria may protect the antibiotic target by gene mutation or modification, and finally, antibiotics can be immediately inhibited by hydrolysis or alteration of their structure (2).
One of the most important weapons in Acinetobacter's arsenal is its amazing genetic flexibility, which allows for quick genetic mutations and rearrangements and the incorporation of external variables carried by the genetic factors into the organism. The most important of them is insertion sequences, which are regarded as one of the most important mechanisms influencing bacterial genomes and eventually evolution. (3).
Different mechanisms play a role in acquiring multidrug resistance (MDR) phenotypes among Acinetobacter baumannii strains (2). The positively charged antibiotics such as Colistin are used mainly against nosocomial pathogens to attach the hydrophilic negatively charged cationic of bacterial LPS, ultimately leading to loss of cellular membrane integrity and cell death (4). However, antibiotic mono-therapy has been identified as a less efficient protocol than combination therapy (5).
This paper aimed to a phenotypic and molecular investigation of multidrug-resistant Acinetobacter baumannii. It included isolating and identifying Acinetobacter baumannii from different clinical infections, determining β-lactams and quinolone resistance, both bla_{VIM} and gyrA genes by using conventional PCR.

Material and Methods

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ISSN: 2776-1010 Volume 3, Issue 6, June, 2022

Sample Collection

(50) Fifty isolates are collected from patients with burn infection who attended Baquba Teaching hospital in Diyala province- Iraq, with different ages (males and females). The collection was done using a swap and kept in the sterile tube at (4) C° and sent to the laboratory to make the culture, biochemical, and molecular detection.

Isolation, Identification, and Susceptibility of Antibiotic

In this research, ten clinical isolates were obtained from burns infections of hospitalized patients (Baquba Teaching hospital in Diyala province- Iraq). Initially, MacConkey agar and Blood agar were used to inoculate samples. Under aerobic conditions, the inoculated agar plates for overnight. VITIC 2 compact system (BioMerieux, France) and IMVIC tests were used to validate bacterial antibiotic sensitivity of A. baumannii isolates. The experiments are carried out in the University of Diyala, Department of Biotechnology, College of Science. Acceptance and written consent were obtained for patients participating and donating samples

Genomic DNA Extraction

The selected genes' detection was listed in (Table 1), DNA extraction was performed using Qigene kit (Germany). Conventional PCR was used with20µl as the final volume reaction mixture to detect the studied genes. Then 5 µl of the PCR reaction was taken and transferred to agarose gel (1.5%) for electrophoresis.

The Used Primers

The used primers are shown in table (1), wherever depended on the NCBI website for providing the database, and primers plus 3 for designing. Table (2) showed temperature, needed time, and cycle number of Denaturation, Annealing, and Extension stages for 16SrRNA, blaVIM, and gyrA gene.

Table (1): sequence of used primers in the study with required temperature for annealing stage and Amplicon size (bp)

Primer Name		Sequences (5/ - 3/)	Annealing temp.	size
blaVIM	F	5`-GATGGTGTTTGGTCGCATA-3`	54	390 bp
	R	5`-CGAATGCGCAGCACCAG-3`		
16S rRNA	F	5`-CAGCTCGTGTCGTGAGATGT-3`	55	150bp
	R	5`-CGTAAGGGCCATGATGACTT-3`		
gyrA	F	5`- AAA TCT GCC CGT GTC GTT GGT-3`	58	343 bp
	R	5`-GCC ATA CCT ACG GCG ATA CC-3`		

Table (2) : PCR Reaction Program

Genes	Denaturation	Annealing	Extension	Cycle
16S rRNA	95°C / 30s	55 °C / 35s	72 °C/40s	30
blaVIM	95°C/30s	52 °C / 40s	72 °C/30s	30
gyrA	95°C/45s	58 °C / 60s	72 °C /45s	30

Statistical Analysis

The graph pad prism V5 program was used to sort all of the data gathered in this report. The desired test was tested on the laboratory request form. A two-way ANOVA test was used. Statistical significance was calculated at a P-value of 0.05.

Results

The current study identified 10/50 (20%) isolates of A. baumannii for burns infections (Figure 1).

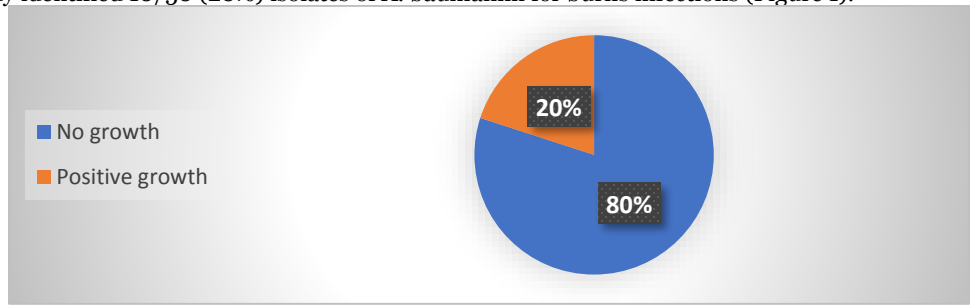

Figure (1): The number of the collected samples.

A. baumannii were Gram-negative under microscopic; their shape varied from bacilli to coco-bacilli (Figure 2). The colonies were non-hemolytic on the blood agar, small, smooth, and creamy appearance. The colonies of clinical isolates were pale pink and had a slight regular edge on MacConkey plates (Figure 3 &4). All isolates were grown at temperatures between 37°C and 44°C.

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ISSN: 2776-1010 Volume 3, Issue 6, June, 2022

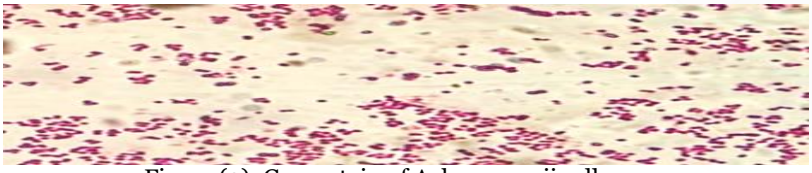

Figure (2): Gram stain of A. baumannii cells

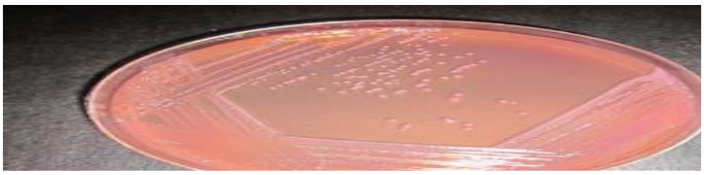

Figure (3): Acinetobacter baumannii colonies on MacConkey agar.

Figure (4): Acinetobacter baumannii colonies on Blood agar (B).

Biochemical Identification

In this study, the initial Identification of A. baumannii using some manual biochemical was summarized in Table (3). All the clinical isolates positively reacted with catalase and Simmons citrate tests and did not produce constant urease. However, all isolates negatively react with the rest tests (Table 3).

Table (3): Initial Identification of A. baumannii

NO	Biochemical tests	Results
2	Catalase production test	+
6	Indole production	-
9	Lactose fermentation	Non-lactose fermentation
3	Methyl red	-
1	Oxidase test	-
4	Simmons citrate	+
8	Triple Sugar–Iron	Alkaline slant /No change bottom, No gas, No H_2S
7	Urease production	Variable
5	Voges- Proskauer	-

After the initial diagnosis by biochemical tests to the isolates, a genotypic method was carried out for the genomic Identification of A.baumannii. PCR results were confirmed by gel-electrophoresis using a 150pb DNA ladder (Figure 5). The 16SrRNA gene is presented in all 10(100%) (Figure 5).

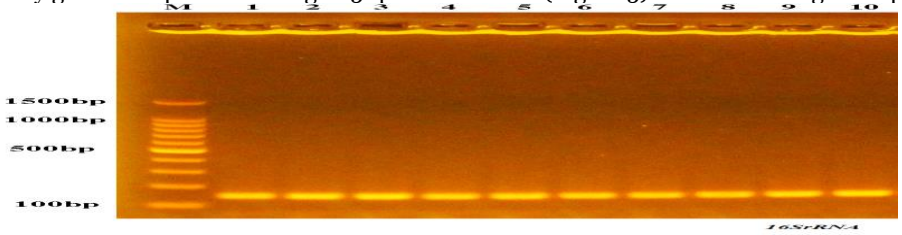

Figure (5): The 16SrRNA amplification for 10 samples of A. baumannii 1.5% agarose gel (90 minutes at 100 volts) electrophoresis using 100bp ladder marker. Lanes 1-10 resemble 150 bp PCR results.

Antibiotics Susceptibility Test

The fifteen antibiotics against A. baumannii showed that all clinical isolates were resistant to most used antibiotics (Table 3). All selected isolates (100%) were entirely resistant to 8 tested antibiotics. Whereas, Imipenem did not affect 90% of A. baumannii. Similarly, only 10% of the isolate were sensitive to Amikacin, Gentamicin, and Tobramycin. However, only 50% of them could resist Trimethoprim-sulfamethoxazole. Finally, all the isolates (100%) could not resist the Colistin, and only 10% showed resistance against Tigecycline and Minocycline, as shown in table (4). The majority of selected isolates resisted β-lactam antibiotic classes; the isolates demonstrated resistance against Imipenem and Meropenem by 90% and 100%, respectively. The findings reveal Ampicillin and Piperacillin's complete

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ISSN: 2776-1010 Volume 3, Issue 6, June, 2022

resistance (Table 2). The whole isolates were susceptible to Colistin (100%), whereas only 15% were susceptible to Tigecycline and Minocycline.

Regarding Trimethoprim /Sulfamethoxazole, we identified that these antibiotics inhibited the growth of 25% of the total tested isolates, as shown in table (2). All selected isolates (100%) were highly resistant to Fluoroquinolones.

Table (4): The percentage of antibiotics resistant by A. baumannii

Antibiotics family	Type of Antibiotics	Sensitive	Resistant
		NO. and %	NO. and %
Penicillins	Ampicillin	0 (0%)	10 (100%)
B-lactam inhibitors	Piperacillin /Tazobactam	0 (0%)	10 (100%)
Cephalosporins	Cefazolin	0 (0%)	10 (100%)
	Cefoxitin	0 (0%)	10 (100%)
	Ceftazidime	0 (0%)	10 (100%)
Carbapenems	Imipenem	1 (10%)	9 (90%)
	Meropenem	0 (0%)	10 (100%)
Aminoglycosides	Amikacin	1 (10%)	9 (90%)
	Gentamicin	2 (20%)	8 (80%)
	Tobramycin	2 (20%)	8 (80%)
Tetracyclines	Tigecycline	9 (90%)	1 (10%)
	Minocycline	9 (90%)	1 (10%)
Fluoroquinolones	Ciprofloxacin	0 (0.0)	10 (100%)
	Levofloxacin	0 (0.0)	10 (100%)
Folate pathway antagonists	Trimethoprim /Sulfamethoxazole	5 (50%)	5(50%)

Eight isolates 8/10 (80%), showed that they have gyrA gene by using PCR, as shown in (Figure 6).

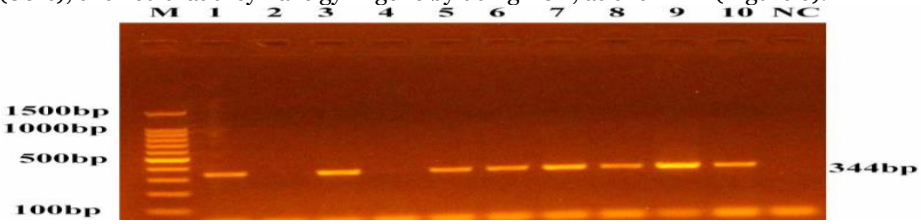

Figure (6): The gyrA amplification for 10 samples of A. baumannii 1.5% agarose gel (90 minutes at 100 volts) electrophoresis using 100 bp ladder marker. Lanes 1-10 resemble 344 bp PCR results.

50% of isolates did not carry the bla$_{VIM}$ gene (Figure 7). Phenotypically, and reveals 90% of resistance against aminoglycosides.

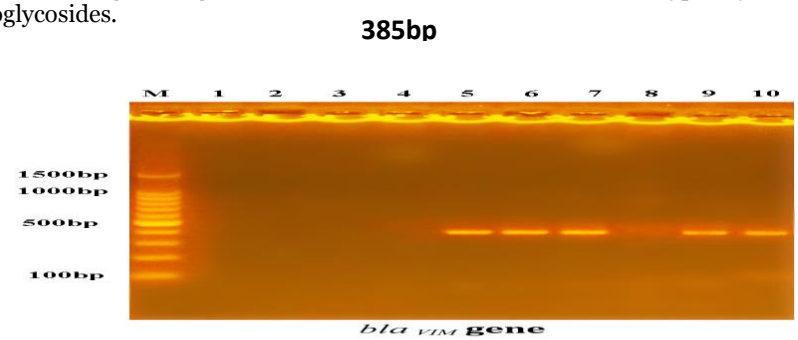

Figure (7): The amplification of bla$_{VIM}$ gene of A. baumannii were emerged on 1.5% agarose gel and stained by Ethidium bromide. 100-1500bp ladder marker. Lanes 1-10 are 385bp PCR products.

All the isolated A. baumannii isolates could form biofilms. Furthermore, the results reveal that all A. baumannii isolates formed a strong biofilm (Figure 8), as the biofilm stained-crystal violet absorbance values ranged from 0.123-0.158 nm.

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ISSN: 2776-1010 Volume 3, Issue 6, June, 2022

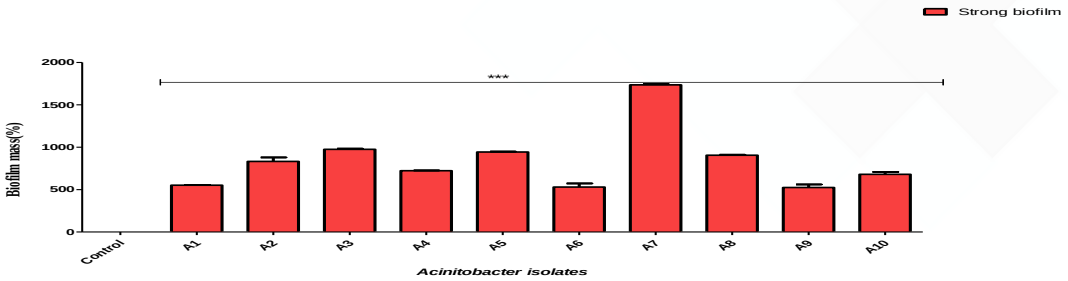

Figure (8): The strength of biofilm formed by the isolated A. baumannii.

Discussion

A. baumannii were Gram-negative under microscopic; their shape varied from bacilli to coco-bacilli. The colonies were non-hemolytic, small, smooth, and creamy on the blood agar. The colonies were pale pink with a slight regular edge on MacConkey agar. The initial Identification of A. baumannii showed a positive reaction for catalase and Simmons citrate without urease production.

The diagnosis by biochemical tests and genotypic method was carried out to identify A. baumannii. The 16SrRNA gene is presented in all the tenth isolates, and that agrees with many local studies using the 16SrRNA gene as an essential gene in the molecular diagnosis of bacterial isolates (7).

The fifteen antibiotics against A. baumannii showed that all clinical isolates were resistant to the most tested antibiotics. All selected isolates (100%) were entirely resistant to 8 tested antibiotics. Imipenem did not affect 90% of A. baumannii. 10% of the isolate were sensitive to Amikacin, Gentamicin, and Tobramycin. 50% of them could resist Trimethoprim-sulfamethoxazole. All the isolates could not resist the Colistin, and 10% showed resistance against Tigecycline and Minocycline.

The majority of selected isolates resisted β-lactam antibiotic classes; the clinical isolates were resistant to Imipenem and Meropenem by 90% and 100%, respectively. A similar study (1) to our results found that A. baumannii was resistant to Carbapenems, Imipenem, and Meropenem (90%).

Our results showed complete resistance to Ampicillin and Piperacillin, similar to the local study that found that all isolates of A. baumannii reveal resistance (Piperacillin/Tazobactam). Furthermore, it was found that all A. baumannii isolates demonstrated high resistance against Ampicillin while 82.4% were resistant to (Piperacillin/Tazobactam) (8). Another study found that 93.8% of A. baumannii survived in the presence of Ampicillin (9, 10).

All isolates were susceptible to Colistin, whereas only 15% were susceptible to Tigecycline and Minocycline. Similarly, (1) and his team (2021) reported that all the isolates of A. baumannii were sensitive to Colistin (11). A. baumanii isolates did not resist Colistin and Tigecycline (100%) (11). Colistin and Polymyxin E are best chosen against multidrug-resistant clinical isolates (9). They have a positive charge that binds to the negative hydrophilic portion of bacterial LPS, resulting in integrity loss of the cellular membrane and killing the pathogenic bacteria (12). Regarding Trimethoprim /Sulfamethoxazole, we identified that these antibiotics inhibited the growth of 25% of the total tested isolates due to dihydrofolate reductases (DHFR and FolA); MDR A. baumannii was resistant to trimethoprim (1).

All isolates were highly resistant to Fluoroquinolones. These results match the study of (13), who found that the percentage of A. baumannii resistance to Fluoroquinolones was (100%). Fluoroquinolones are broad-spectrum antibiotics that prevent a wide variety of bacterial infections. In addition, they are significantly contributing to the rapid increase of bacterial resistance over the past years (9).

Eight isolates (80 %) displayed gyrA gene. The quinolones resistance is mainly driven by gyrA gene. It causes the change in phenotypic DNA gyrase end topoisomerase IV, leading to antibiotic affinity reduction (14). Half the selected isolates did not carry the blaVIM gene. Phenotypically, the bacterial isolates displayed 90% of resistance against aminoglycosides. These findings were closely related (1), who found that all selected clinical isolates were resistant to Gentamicin and Amikacin (1). Resistance to β-lactam antibiotics is often related to β-lactamases production, which catalyzes the hydrolysis of the amide bond in the β-lactam ring and modifies the antibiotic to an inactive form. Furthermore, bacterial resistance to -lactams might be caused by mutations in penicillin-binding proteins, alterations in external membrane porins (resulting in lower permeability), and the evacuation of antibiotics from the cell via an efflux pump, among other things (15).

All the isolated A. baumannii isolates could form biofilms in the current study. These were in agreement with the study of (8), who found that all (100%) of A. baumannii isolates had the ability to biofilm formation.

Our study showed that all A. baumannii isolates formed a strong biofilm, as the biofilm stained-crystal violet absorbance values ranged from 0.123-0.158 nm. A similar study found that all bacteria's clinical isolates formed strong biofilms on abiotic surfaces (13).

Many bacterial pathogens, including A. baumannii, can form matrix-enclosed communities, referring to biofilm formation. The potential for biofilm formation by A. baumannii isolates possibly plays an essential role in the survival and persistence of bacterial infection in environmental stress factors (11).

The virulence factors of A. baumannii, including efflux system, biofilm-associated protein, pili motility, and quorum sensing system, are included in biofilm formation and associated with the pathogenicity of A. baumannii (16). Extracellular polymeric substances (EPS) in biofilms are responsible for cell-cell and cell-surface binding and participate in the development of biofilm structure and maturation (9).

Conclusion

ISSN: 2776-1010 Volume 3, Issue 6, June, 2022

A. baumannii showed high resistance to most tested antibiotics but was highly sensitive to Colistin. The Majority of A. baumannii is generated strong biofilms for increased colonization.

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