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DETECTION OF THE SUSCEPTIBILITY TEST, BIOFILM FORMATION IN ACINETOBACTER BAUMANNII ISOLATED FROM DIFFERENT SOURCES IN KIRKUK CITY

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Abstract:
This research presents the isolation of Acinetobacter baumannii from different clinical sources such as burns, wounds, sputum and blood. A total of 10 isolates from the studied bacteria were collected and identified using microscopic examinations, culture, biochemical tests, and final diagnosis using the API 20 E system, as well as the use of polymerase chain reaction to detect the presence of the 16srRNA gene to confirm and identify Acinetobacter spp (molecular methods) by PCR. The bacteria showed growth at different temperatures until at 44°C. Antibiotic sensitivity test for 12 antibiotics was carried out using the Kirby-Bauer publication method. The results showed that the isolates were highly resistant to antibiotics, so that the isolates were 100% resistant to Ampicillin, Amoxicillin, Piperacillin, chloramphenicol, cefotaxime, Amoxicillin-Clavulanc acid, Amikacin And resistance to Tetracycline antibiotic by 90% and resistance to antibiotics Ceftazidime, Lefofioxacin, Trimethoprim-Sulphamethoxazole by 80%, while the most effective antibiotic against isolates was imipenem, which gave sensitivity by only 40%.
Some virulence factors were examined, such as the ability of A.baumannii bacteria to form biofilms using the microplate method. The percentage of biofilm-forming isolates was 8/10 (80%), and the isolates were divided into four groups, 2 (20%) non-cell membrane-forming, 1 (10%)) Biofilm formation was strong, 5 (50%) was moderate, 2 (20%) was weak.
The DNA of A. baumannii isolates was extracted, and the csuE gene, which has a role in the formation of biofilms, was detected by PCR. Gel electrophoresis showed that the csuE molecular weight was 168 base pairs, and the percentage of isolates carrying the csuE gene was 100%.

Keywords:

INTRODUCTION

A.baumannii is a non-glucose fermenting Gram-stain-negative opportunistic pathogen that causes severe, difficult-to-treat infections. The infection rate of A.baumannii has been increasing year by year in human medicine and it is also considered as a major cause of nosocomial infections worldwide. These bacteria, also known for their ability to form biofilms, have a strong ability to adapt to the environment and have multidrug resistance (MDR) ([1]). They are non-motile and the name of this genus comes from the Greek word kinetos-bacter.) which means a non-motile rod, although they exhibit rod morphology and twitching motion. To date, the novelty of molecular methods has correctly identified more than 65 published species within the genus Acinetobacter

[1] Nocera et al., 2021

([2]). Carbapenem-resistant A.baumannii has been identified as a critical pathogen due to its high resistance to antibiotics, and has set an alarm for infections caused by this bacterium. According to the organization's report, A.baumannii becomes a major public health threat when it acquires resistance to widespread antibiotics such as carbapenems ([3]) A.baumannii multidrug resistance (MDR) is attributed to widespread misuse of antibiotics and poor oversight.

MDR isolates are associated with a medical history of long hospital stays, the presence of catheters, and mechanical ventilation ([4]). Hospitals ([5]) cause pneumonia and septicemia in immunocompromised patients and also persist for a long time in harsh environments (walls, surfaces, medical devices) in hospital settings ([6]) There are many factors Virulence involved in biofilm formation in A. baumannii such as outer membrane protein A (OMPA), biofilm associated protein (Bap), and the filaments encoded by chaperon-usher pilus ([7]) The ability of biofilm formation contributes to the easy survival and transmissibility of A.baumannii bacteria in the hospital environment, such as its attachment to various biotic and abiotic surfaces ([8])

MATERIALS AND METHODS:

Sample collection and separation:

10 isolates were obtained from different clinical samples of urine and sputum samples from the respiratory tract, burn and wound swabs, blood samples and meningitis samples from Kirkuk governorate hospitals during the period from November 2021 to March 2022. Samples were inoculated on culture media with blood agar and MacConkey Agar and incubated at 37 °C for 24 hours. The isolates were identified using phenotypic examination according to their colour, size and shape of the developing colonies and were identified by microscopy (Cram stain) and biochemical tests including catalase test, oxidase test, urease test and IMVIC test including: indol, methyle red, voges-proskauer and citrate). A.baumannii was also diagnosed using the API 20E system (BioMérieux, France), and partial identification of A.baumannii was also used by detecting the 16srRNA gene.

Antibiotic sensitivity test:

Ten isolates of A.baumannii bacteria were tested for sensitivity using 12 antibiotics using Kirby-Bauer method. These antibiotics included Amoxicillin, Ampicillin, Piperacillin, Chloramphenicol, Cefotaxime, Amoxicillin-Clavulanic acid, Amikacin, Trimethoprim-Sulphamethoxazole, Ceftazidime, Levofloxacin, Tetracycline, Imipenem, most of which are commonly used in hospitals in Qatar to treat various infections. The results were compared with the National Committee for Clinical Laboratory Standards. ([9]) to define as resistant, moderate or sensitive.

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[2] Sarshar et al., 2021
[3] WHO., 2017
[4] Kyriakidis et al.,2021
[5] Ramirez et al., 2020
[6] Pourhajibagher et al., 2016
[7] Csu Thummeepak .at.el.2016 & Ghasemi .et.al.2018
[8] Yang.et.al.20 19
[9] CLSI. 2018

Detection of biofilms using the microtitration plate method.

The microtiter plate method was used to detect biofilm formation of A.baumannii bacteria. Biofilm formation was identified by the dye crystal violet, whose color intensity is directly related to the biofilm concentration. An Enzyme Linked Immunosorbent Assay ELISA was used to measure the absorbance at 630 nm ([10]).

DNA extraction

Bacterial isolates were grown on brain heard infusion broth and incubated at 37 °C for 24 hours, and DNA was extracted from A. baumannii using the Presto™ Mini g DNA Bacteria Kit by Company (Geneaid, Taiwan).

Molecular detection of the 16s rRNA gene

Isolates were identified at the genus level using a gender-specific 16SrRNA PCR assay, the 16srRNA gene was amplified with a size of 240 base pairs using the primers F (5′-TTTAAGCGAGGAGGAGG -3′) and R (5′- ATTCTACCATCCTCTCCC -3). DNA from each isolate was extracted by a genomic DNA extraction kit (Geneaid, Taiwan) and used as a template. PCR assays were performed using a PCR Master Mix (Promega, USA) in a PCR thermal cycler (LifeECO, chain). PCR was performed in a 25 µl reaction volume and consisted of 3 µl of genomic DNA, 12.5 µl of mix PCR Master mix, 1 µL of F-primer, 1 µL of R-primer, and 7.5 µL of deionized water ddH2O, and the reaction steps were as follows: denatured at 95 °C for 4 minutes.

Followed by 35 cycles of secondary denaturation at 95 °C for 30 seconds, annraling at 58 °C for 45 seconds, extension at 72 °C for 45 seconds, and final extension at 72 °C for 5 minutes.

Molecular detection of a causE gene related to biofilm formation:

Polymerase chain reaction (PCR) assays were performed to detect the csuE gene by primer F:5-CATCTTCTATTTCGGTCCC-3 and reverse primer R:5-CGGTCGATGAGCATTGGTAA-3 of size 168bp and DNA was extracted from each isolate by a genomic DNA extraction kit (Geneaid, Taiwan).) and use it as a template. PCR assays were performed using a PCR Master Mix (Promega, USA) in a PCR thermal cycler (LifeECO, chain). PCR was performed in a 25 µl reaction volume and consisted of 3 µl of genomic DNA, 12.5 µl of PCR Master mix, 1 µl of F-primer, 1 µl of R-primer, and 7.5 µl of deionized water ddHO . The PCR conditions were primary denaturation at 95°C for 4 minutes, followed by 35 cycles of secondary denaturation at 95°C for 30 seconds, gene annealing temperature of 59°C (binding of primers with template DNA) for 45 seconds, and extension at 72°C. for 45 minutes, followed by final extension at 72°C for 5 minutes.

Electrophoresis process:

PCR results were electrophoresed by 1% agarose gel with 3 µl of Safe Red dye in XSB1 solution using a DNA ladder 100–1500 bp (Bioneer, Korea) at 90 V for 80 min, and DNA was scanned and imaged using UV. Violet. ([11])

RESULTS AND DISCUSSION:

[10] Tang et.al., 2011
[11] Sambrook and Russell 2001

Isolates were collected from patients with burns, wounds, sputum and blood samples, and ten isolates of A.baumannii were identified out of 150 isolates. Regardless of the total number, more burn isolates were obtained, followed by wounds and then blood and sputum samples. The isolates were diagnosed based on routine examinations such as microscopic examination, cultural characteristics, and biochemical tests as in Table (1), where the sex was diagnosed at first, then the species was confirmed using API 20E, as well as using polymerase chain reaction to detect the presence of the 16srRNA gene to confirm and identify Acinetobacter spp. (Molecular methods) by PCR where all samples were 100% carrier of the gene after migration on the agarose gel as in Figure (1)

Table 1: Results of biochemical tests.

Examination	The result
lactose fermentation	-
Gram stain	-
Hemolysis	-
Indole assay	-
Oxidase assay	-
Motion check	-
Fox_proscar	-
Ureas test	-
Instance red check	-
Red blood cell lysis	-
H2S production	-
Catelize examination	+
A growth on the center of the blood vessels	+
Growth at 44 °C	+
Jacket consumption check	+
Examine the consumption of triglycerides	MSD/ no color change

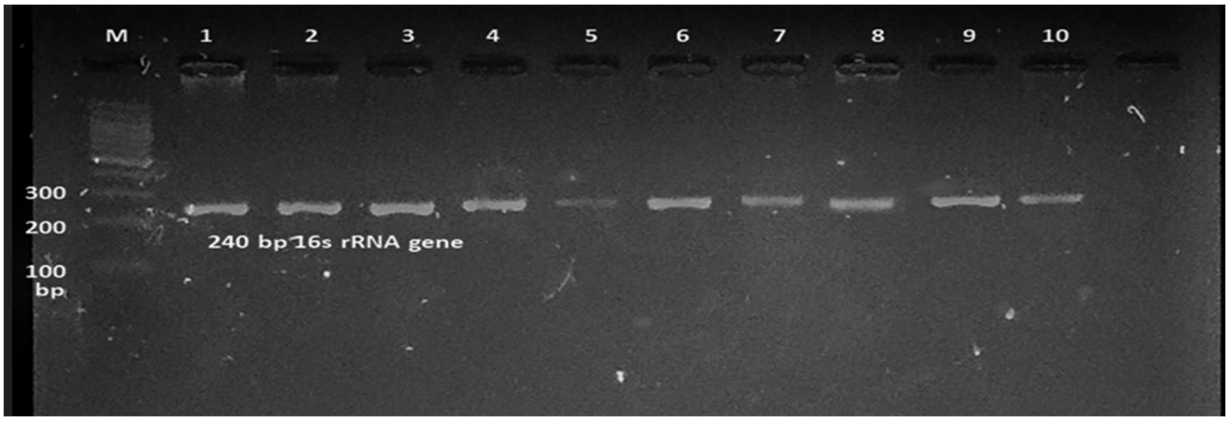

Figure (1) Electrophoresis of the 16srRNA gene of A.baumannii bacteria on an agarose gel with a concentration of 1% containing 0.3 µg/ml of the safe red dye using a DNA ledder at a voltage of 75 volts/cm for an hour. It was found that all pathways contain the gene.

Among the newly identified pathogens, Acinetobacter baumannii plays an important role in the colonization and infection of hospitalized patients. They have been implicated in a variety of nosocomial infections, including bacteremia, urinary tract infections, secondary meningitis, and wound and burn infections but their predominant role is as agents of nosocomial pneumonia, especially ventilator-associated pneumonia in patients confined to intensive care units (ICU's). . Treating such infections is often very difficult for a clinician because of widespread resistance of the organism to a large number of antibiotics ([12]).

A. baumannii is the second most common cause of multidrug resistance (MDR) in nosocomial infections of burn patients. Infection is recognized as the most common cause of death for burn injuries. Infection has become a serious challenge for health care systems due to the increase in resistance to antibiotic agents in nosocomial isolates of A. baumannii, mostly Incorrect administration of antibiotic therapy for an infection leads to the emergence of widespread drug-resistant (XDR) strains that pose significant health challenges by prolonging recovery time, treatment failure, and increased mortality ([13]). It is clear that burn patients are at greater risk. They are prone to infection in general, and nosocomial infections in particular, mainly due to the immunocompromising effects of burns, long hospital stays, and intensive diagnostic and therapeutic procedures ([14]).

Antibiotic sensitivity of A.baumannii

In the antibiotic sensitivity test, the results showed that the isolates were highly resistant to antibiotics, so that the isolates that were 100% resistant to Ampicillin, Amoxicillin, Piperacillin, chloramphenicol, cefotaxime, Amoxicillin-Clavulanc acid, Amikacin, and 90% resistant to the antibiotic Tetracyline were resistant to antibiotics. Ceftazidime,Lefofioxacin,Trimethoprim-Sulphamethoxazole by 80%, while the most effective antibiotic against the isolates was Imipenem, which gave a sensitivity of only 40%. Due to the high resistance to its ability to produce beta-lactamase enzymes such as broad-spectrum beta-lactamase enzymes, an enzyme that breaks the beta-lactam ring in the antibiotic, which makes it inactive and makes Acinetobacter baumannii resistant to antibiotics that contain the beta-lactam ring. In many bacteria, which leads to multiple resistance to different antibiotics, in addition to modifying the structure of penicillin-binding proteins (PBPs), which are a major target for beta-lactam antibiotics ([15]).

Imipenem is the optimal and effective treatment for A. baumannii. In a local study conducted in 2006 by Al-Khafaji, it was revealed that clinical A. baumannii isolates were completely sensitive to Imipenem, and in another study conducted on isolates of American patients in US health institutions to treat infected soldiers returning from Iraq. The isolates were sensitive to Imipenem by a large percentage of up to 90% compared to other antibiotics ([16]). However, in the past few years, the percentage of A.baumannii

[12] Donlan, & Costerton, 2002
[13] Farshadzadeh.et.al.2015
[14] Alaghehbandan.et.al.2012
[15] Mussi.et.al.2005
[16] Scott.et.al.2007

resistance to this antibiotic has increased due to the excessive use of these antibiotics. In addition to In addition, the spread of resistant strains among hospitals in the absence of strict infection control measures.It was found that resistance to carbapenem is associated with a high mortality rate and resistance to other classes of antibiotics such as aminoglycosides and quinolones ([17]). There are multiple resistance mechanisms, and the most common mechanism is its ability to produce Carbapenem hydrolyzing class D β-lactamases (CHDLs) that destroy carbapenems ([18]). Others, such as the production of carbapenemases, which belong to beta-lactamase enzymes, and their ability to form a biofilm ([19]).

This study identified differences in antibiotic resistance. The results indicated high resistance to the antibiotics used at different rates, as the antibiotics (100%) resisted ampicillin and cefotaxime, followed by amikacin and ciprofloxacin at 83%, then the antibody gentamicin at 58%, and finally imipenem at 50%. %. These results are consistent with what Suhail 14 mentioned, as their study showed that isolates of this species gave the lowest resistance to imipenem by 5-19%.

The reasons for the resistance of these bacteria to penicillin may be due to the widespread and indiscriminate use of these antibiotics, as well as the transmission of resistance through plasmids or transposons. Acinitobacter baumanii strains have the potential to rapidly acquire antimicrobial resistance as newer antimicrobial resistance has been reported worldwide. In general, resistance to the group of cephalosporins is due to the bacteria having multiple resistance mechanisms and their production of beta-lactamase enzymes and that they have the ability to change the outer membrane proteins 15. The resistance of bacteria to the third generation cephalosporins (Cefotaxime, Cefixime, Cefriaxone) is linked to their indiscriminate use, which leads to the development of their resistance. Another reason is its production of broad-spectrum beta-lactamase enzymes as well as the production of AMPK-type beta-lactam enzymes that inhibit the action of third and fourth generation cephalosporins. Resistance to aminoglycosides arose from the possession of plasmids that encode the aminoglycoside-modifying enzymes represented by the 30s small ribosomal subunit, thus preventing them from binding to ribosomes. The antibiotic imipenem was the optimal and effective treatment for these bacteria. It inhibits transpeptidase activity and stimulates cell rupture. During the past few years from 2010-2018, the resistance of the bacteria under study to this antibiotic has increased, and the reason for this is that it has several resistance mechanisms, perhaps the most common of which is Carbapenem-hydrolyzing class D beta lactamase. CHDLs that break down carbapenems also break down a protein in the outer membrane known as the core associated with resistance against these 16 antibiotics.

Table (2) Percentages of isolates of Baumannii bacteria that are resistant, intermediate and sensitive to antibiotics

NO	name Antibiotic		Resistance isolates	Medium resistance	Sensitive isolates
1	Amoxicillin	AX	10(100%)	0(0%)	**0(0%)**

[17] Abd El-Baky.et.al.2020
[18] Lee.et.al.2011
[19] Mussi.et.al.2005

2	Ampicillin	AM	10(100%)	0(0%)	**0(0%)**
3	Piperacillin	PRL	10(100%)	0(0%)	**0(0%)**
4	Chloramphenicol	C	10(100%)	0(0%)	**0(0%)**
5	Cefotaxime	CTX	10(100%)	0(0%)	**0(0%)**
6	Amoxicillin-Clavulanic acid	AMC	10(100%)	0(0%)	**0(0%)**
7	Amikacin	AK	10(100%)	0(0%)	**0(0%)**
8	Ceftazidime	CAZ	8(80%)	1(10%)	**1(10%)**
9	Tetracycline	TE	9(90%)	1(10%)	**0(0%)**
10	Trimethoprim-Sulphamethoxazole	SXT	8(80%)	2(20%)	**0(0%)**
11	Levofloxacin	LEV	8(80%)	0(0%)	**2(20%)**
12	Imipenem	IMP	6(60%)	1(10%)	**3(30%)**
Mean ± S E			9.083 ± 0.37	0.416 ± 0.19	**0.5 ± 0.28**

ANOVA analysis showed that there was a significant difference between the variables, p@0.05 = <0.001

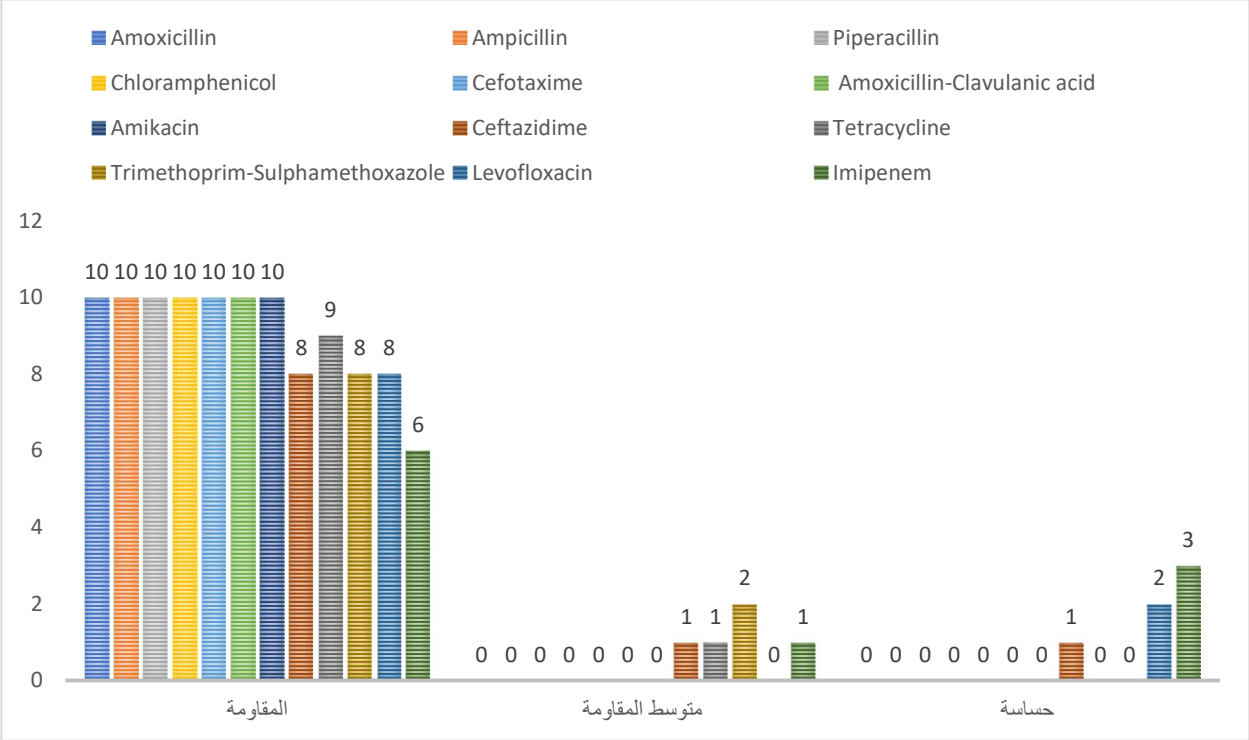

Figure (3) Percentages of isolates of A.bumannii bacteria that are resistant, intermediate and sensitive to antibiotics.

The ability of A.baumannii isolates to form biofilms

Of the total of 10 A.baumannii isolates capable of forming biofilms, the proportion of biofilm-forming isolates was 8/10 (80%). The isolates were divided into four groups: 2 (20%) did not form cell membranes, 4 (40%) biofilm formation was strong, 3 (30%) was moderate, and 1 (10%) was weak. This is consistent with a local Iraqi study ([20]) and ([21]) the formation of biofilms enhances its survival in the hospital environment, as well as enhances antibiotic resistance, as biofilms act as a barrier that protects bacteria from antibiotics.

Detection of the biofilm-related csuE gene of A.baumannii bacteria using a PCR thermal cycler.

A biofilm-associated gene, csuE, which encodes filaments and contributes to biofilm formation, which is possessed by A.baumannii, was detected. Molecular analyzes in this study showed that all A.baumannii isolates contain the csuE gene as shown in the figure (4). PCR for all A.baumannii isolates was done using primers specific to the csuE gene for the purpose of detection. The results of the investigation showed that this gene has a molecular weight of 168 pairs. Basis is close to the results obtained by ([22]) and close to an Iranian study ([23]) where the isolates carried the csuE gene by (100%) and another Taiwanese study ([24]) isolates carrying the csuE gene by (91.6%).

[20] Al- Zubaidi.2020
[21] AL-Taati.et.al.2018
[22] Al-Zubaidi.2020
[23] Zeighami et.al.2019
[24] Yang et.al 2019

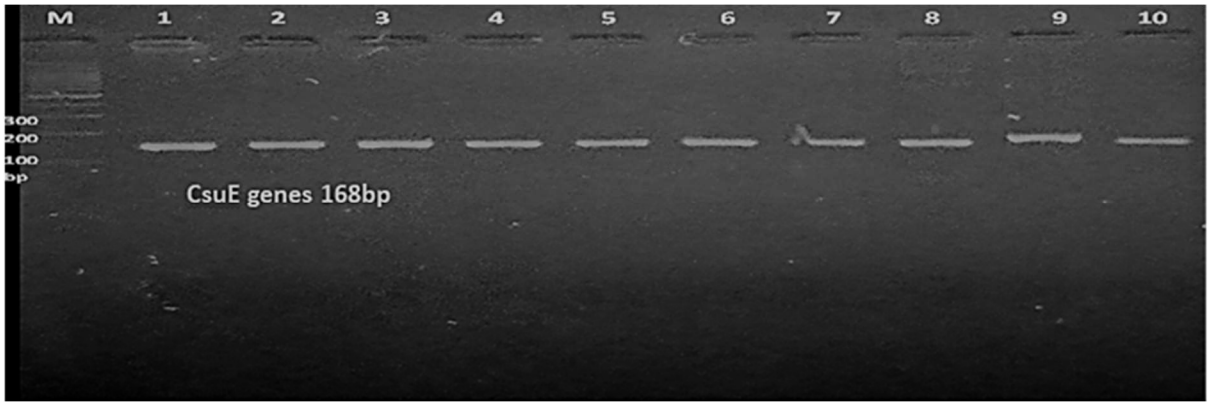

Figure (4) Electrophoresis of the csuE gene in A.baumannii bacteria on an agarose gel with a concentration of 1% containing 0.3 µg/ml of safe red dye using DNA ledder at a voltage of 75 V/cm for an hour.

CONCLUSION:

The diagnosis of bacteria by means of 16srRNA molecular methods is the best way to detect the bacterium A.baumannii, and this bacterium has a great ability to form biofilms, which contributes to increasing the bacterial resistance to antibiotics, as well as excessive use of antibiotics that also causes high resistance to antibiotics. It is preferable to study more about biofilms and expression Its genome, like baP and ompA, encodes a protein associated with bacterial biofilms that may be a target for modern antibiotics.

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