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Research Article

Isolation and identification of multi-drug resistance *Acinetobacter baumannii* isolated from clinical samples at Baghdad, Iraq

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Abstract

An opportunistic bacterium called *Acinetobacter baumannii* has significantly increased the frequency of infections in recent years. With only a limited number of "traditional" virulence factors, It is infections have spread rapidly through hospitals across the globe. The present study aimed to work out the relationship between the multi-drug resistance (MDR) of *A. Baumannii* and biofilm formation. A total of 150 samples were collected from various clinical sources from different age groups and gender patients in Ghazi AL Hariri Hospital and Baghdad Teaching laboratories in Medical City in Baghdad, Iraq from December 2021 to March 2022. Microscopical inspection and cultural features on various culture media, including culturing on selective medium CHROMagar, were used to identify bacterial isolates. The characteristics of the isolates were then established by some biochemical tests. Identification was confirmed using the Vitek-2 system with an accuracy of 99%, which revealed that only (50) isolates were given identical morphological characteristics and biochemical tests belonging to *Acinetobacter baumannii* isolates. 28 (56%) isolates were collected from burns. 10 isolates (20%) and 9 isolates (18% were collected from wound and sputum cultures of *A. baumannii* , respectively, while only 3 isolates (6%) were from urine culture. The susceptibility test for all the fifty clinical isolates of *A. baumannii* was performed against 10 different antibiotics. The results showed that *A. baumannii* isolates were Multidrug-resistant (MDR) (98%), while the other (2%) of the isolates were extensively drug-resistance (XDR) to the majority of antibiotics tested. All 50 isolates in the present study were subjected to the micro-titer plate (MTP) assay method (96 walls). The results indicated that strong biofilm was detected in 40 (80.0%) of the tested isolates. Thirty bacterial isolates were were found to be MDR and had strong biofilm production.

Keywords: *Acinetobacter baumannii,* Antibiotic susceptibility, Biofilm production, 16srRNA

INTRODUCTION

Acinetobacter genus is s a non-motile, aerobic, pleomorphic, and gram-negative coccobacillus. Infections brought on by pathogenic members of the species Acinetobacter are becoming a serious danger to human health because they are opportunistic pathogens. (Peleg and Hooper, 2010). *Acinetobacter baumannii* is responsible for numerous local and general infections, such as asthma, septicemia, and wound infections, illnesses contracted in hospitals that are drug-resistant *A. baumannii* spread rapidly throughout the globe, primarily in critical care units (Wieland *et al.,* 2018).

This pathogen can infiltrate host cells and survive there. It initially attaches to host cells before invading and moving into the nucleus. It spreads throughout organs and the bloodstream after destroying host cells. Its locations of infection include the respiratory tract, blood, pleural fluid, urinary tract, surgery incisions, central nervous system, epidermis, and eyes. Nosocomial pneumonia and bacteremia are the two clinical symptoms of *A. baumannii* types that occur most frequently (Parra-Millan *et al.,* 2018). The *A. baumannii* strains with a biofilm-forming ability that can produce biofilms on the surface of the endotracheal tube may pose a serious concern to those patients who require mechanical ventilation. Consequently, this can lead to relatively high levels of colonization in the lower part of the respiratory tract (Corneliu *et al.,* 2021).

A crucial component of Gram-negative bacteria's outer

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membrane proteins (OMPs), outer membrane protein A (OmpA) is a critical virulence factor that regulates the development of bacterial biofilms, eukaryotic cell infection, drug tolerance, and immunomodulation (Dan *et al.,* 2020). OmpA works with external matrix proteins like fibronectin to assist epithelial cells in adhesion and eventual penetration. OmpA attaches to factor H in human blood, helping *A. baumannii* avoid being killed by complement. Additionally, the loss of OmpA lowers the minimal inhibitory concentrations (MICs) of numerous antibiotics, such as chloramphenicol and aztreonam, suggesting that OmpA is essential for expulsion of antibiotics from bacterial cells, which results in the Multidrug-resistant (MDR) phenotype of *A. baumannii* (Mary, 2022). Bacteria can survive in comparatively harsh conditions because of their capacity to produce biofilms. In hospitals, bacteria adhering to fake surfaces promotes long-term toughness, endurance for dry environments, and utilization of different metabolic resources. These characteristics make it nearly difficult to eradicate biofilm-associated bacteria from the medical surroundings (Babapour *et al.,* 2016). The present study aimed to study the relationship between the MDR phenotype and biofilm formation.

MATERIALS AND METHODS

Ethical approval

This study was approved by the ethical committee of the Biotechnology Department College of Science / Al-Nahrain University (Reference No.: CSEC/0121/0010).

Sample collection

This study was conducted during the period from December 2021 to March 2022. A total of 150 clinical samples were collected (respiratory tract secretion (sputum), burins, wounds swaps and urine) from patients from Ghazi AL Hariri Hospital and Baghdad Teaching laboratories in Medical City in Baghdad/ Iraq. The samples were taken by sterile disposable cotton swabs from burins and wounds while sputum and urine were taken from sterile disposable caps by transport medium.

Table 1. Antibiotic discs used for the study

No.	Antibiotic discs	Symbol	Con. (µg / disc)	Origin
1	Amikacin	AK	30 µg	
2	Amoxicillin	AX	25 µg	
3	Ciprofloxacin	CIP	5 µg	
4	Colistin	CL	10 µg	
5	Gentamicin	GN	10 µg	Liofilchem /
6	Levofloxacin	LEV	5 µg	(Italy)
7	Polmyxin	PB	300 µg	
8	Vancomycin	VA	30 µg	
9	Azithromycin	AZM	15 µg	
10	Aztreanam	ATM	30 µg	

Identification of bacteria isolates

All collected clinical samples were cultured on CHROMagar, MacConkey agar and Blood agar and then incubated at 37 °C for 24 hrs. Each bacterial isolate was identified by using several morphological, microscopical and biochemical tests (Oxidase, Catalase, Indol and Simmon Citrate test). Vitek-2 system was used to accurately identify each bacterial isolate (99%). Then isolates were confirmed by molecular identification by using 16srRNA gene.

Antibiotics susceptibility test

This test was achieved by the disk diffusion method for 10 different antibiotics (Table 1) according to the Clinical and Laboratory Standards Institute guideline (CLSI) 2021 as per the following guideline (Hudzicki, 2009).

Colonies were moved to 3 cc of ordinary saline from an overnight Muller-Hinton agar plate culture. A 0.5 McFarland adjustment, or 1.5108 CFU/ml, was made to the turbidity. The bacterial suspension was applied using a clean cotton swab, and any extra liquid was squeezed out by pushing the brush against the tube wall. After being seeded onto Muller Hinton agar plates, the bacterial solution was allowed to dry for 15 minutes. Different antibiotic discs were used, and a maximum of six discs were put on the medium's surface using sanitized tools and a small amount of pressure to secure the disk in place. After that, the dish was kept for 24 hours at 37 °C. Testing was done on the 10 antibacterial discs.

Detection of biofilm formation

Biofilm formation of *A. baumannii* was quantified by the micro-titer plate assay method (96 walls). It was used to assess the ability of bacterial isolates to form biofilm, as mentioned by Almeida *et al.* (2013) as follows:

Bacterial isolates were activated by growing them on brain heart infusion (BHI) at 37 ºC for 24 hrs. A volume of 20 µl of the bacteria suspension was transferred to the Eppendorf tube containing BHI broth and glucose and compared with a 0.5 McFarland tube. The last line of wells (12 wells) contained 200 µl of BHI broth with glucose (0.25 %) only with no bacteria and was considered as negative control (NC). While 200 µl of bacterial suspension on Eppendorf was transferred to the other wells of the micro-titer plate, 3 triplicate was done for each isolate. The covered micro-titer plate was sealed with Para-film during incubation at 37 ºC for 24 hrs. By washing the wells three times with normal saline, unattached bacterial cells were removed and then left to dry at room temperature for 10 min. Then a volume of 200

Table 2. Classification of bacterial isolates according to OD of NC in micro-titer plate assay method.

Mean Optical Density	Adherence Biofilm
ODc.*< ODA* < ODc.×2	Non adherent / Weakly adherent
ODc×2 < ODA < ODc×4	Moderately adherent
ODc×4 < ODA	Strongly adherent

*ODc.= Mean optical density of negative control;*ODA= Mean of three optical densities of each *A. baumannii* isolate; NC: Negatuve control

Table 3. Reaction mixture of PCR

Component	16srRNA Gene
Master-mix	5 µl
Forward primer	1 µl
Revers primer	1 µl
DNA template	3 µl
Deionized water	15 µl
Total	25 µl

Table 4. Program PCR amplification of 16srRNA gene

Steps	Temperature (ºC)	Time	Cycle Number
Initial Denaturation	95 ºC	5 min.	1
Denaturation	95 ºC	30 sec.	
Annealing	51 ºC	30 sec.	35
Extension	72 ºC	1 min.	
Final Extension	72 ºC	5 min.	1

µl of methanol was added to plate wells for 15 min. (for fixation of the bacteria on the surface of the wells). After 15 min, it was removed and the plate was left to dry at room temperature. The wells were filled with 200 µl of crystal violet dye for 30 min. After removing the crystal violet solution, wells were washed three times with distilled water to remove the unbounded dye and allowed to dry at room temperature for 10 min. Plate was then placed in the ELISA reader after the dye attached to the adherent cells was re-solubilized with 200 µl of pure ethanol. . By using an ELISA reader with a frequency of 630 nm, the marked adherent bacteria's optical density (OD) was calculated. (OD 630nm). These OD values were used as a gauge for how well microbes adhered to surfaces and developed biofilms. Based on the OD values found for each isolate of *A. baumannii*, the categorization listed in Table 2 was used to calculate the data.

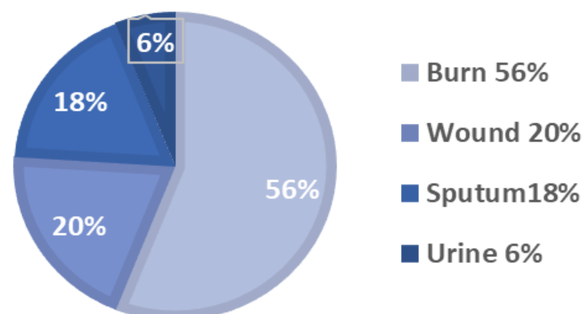

Fig. 1. *Isolates of Acinetobacter baumannii are distributed according to the sample type*

DNA extraction

Clinical isolates of *A. baumannii* had their genomic DNA extracted using a commercial purification system (BIONEER/Korea), and their purity and concentration were measured using a spectrophotometer (Nanodrop) instrument. The genomic DNA was then quantified and stored at -20°C.

Primer

16srRNA primer F:5´ AGAGTTTGATCCTGGCTCAG 3´ and R: 3´ TACCTTGTTACGACTT 5´ (Si *et al.,* 2010) were obtained in lyophilized form. The stock solution was prepared by suspending lyophilized primer in nuclease-free water, and the amount of water was based on the information sheet supplied. A volume of 10µl from the Stock was added to 90µl of nuclease-free water to prepare a working solution.

PCR Reaction mixture

As shown in (Table 3), a Polymerase chain reaction (PCR) mixture was made with a total amount of 25 l per reaction, 1 l forward and reverse primers, 5 l of premix (master mix), 15 l of Deionized water and 3 µl of *A. Baumannii* DNA was added to the mixture, the PCR tube was then moved to the thermal cycler, where the protocol for each primer's amplification was begun. The amplification products were then examined on a 2 % agarose gel in the presence of a 1500-bp DNA ladder marker.

Table 5. Negative and positive growth according to the site of collection

Type of sample	No. of samples	No. of positive growth samples	No. of negative growth samples
Burn	53	35	18
Wound	49	30	19
Sputum	35	14	21
Urine	13	7	6
Total No. (%)	150 (100%)	86 (58%)	64 (42 %)

RESULTS AND DISCUSSION

Collection of samples

The results revealed that only 50 isolates were A. baumannii and shared the same morphological traits and molecular assays. The other (36) isolates seemed to be related to different pathogenic genera. Most of them were *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, and *Enterococcus faecium* and the rest 64 (42%) were negative samples. In this work, out of 50 positive cultures for *A. baumannii*, 28 (56%) isolates were collected from burns. This result agreed with the work of Raghda *et al.* (2018) which have a near result (54%). While wound and sputum cultures of *A. baumannii* were 10 isolates (20%) and 9 isolates (18%), respectively, and this result was similar to the work of Sura *et al.* (2018), who showed a near percentage (17.3%) and (16%) for wound and sputum respectively. On the other hand, there were only 3 isolates (6%) of urine culture and this percentage was near to the percentage of the work of Ali and suhad (2021), which was 4% for urine culture for *A. baumannii* (Fig. 1, Table 5).

Identification of bacterial isolate

Cultural Characteristics

The morphological identification was confirmed depending on the characteristics of colonies grown on selective synthetic media CHROMagar, blood agar and MacConkey agar.

Due to the substantial background flora in gathered specimens, it may be challenging and time-consuming to identify *A. baumannii* using conventional culture media, particularly when using media based on separation by the lactose/non-lactose fermentation ability. These challenges were surmounted by using CHROMagar Acinetobacter, a highly selective medium for quick detection that allowed the development of Acinetobacter in glaringly red colonies after overnight incubation (Moran-Gilad *et al.,*2014). After 24 hours and incubation at 37 °C, *A. baumannii* isolates on the CHROMagar showed vibrant crimson colonies. Enzymatic substrates were incorporated into CHROMagar., allowing for early colour-based recognition of colonies found within 18 to 24 hours of inoculation. Additionally, this medium includes substances that prevent the majority of Gram-positive and -negative microbes from growing (Ciftci *et al.,* 2015). On the other hand, *A. baumannii* colonies emerged on MacConkey agar as a non-lactose fermenter, with masses of solitary circular colonies that were 1-2 mm in diameter and transparent colonies (Paymani *et al.,* 2011). While on blood agar, colonies appeared grey in color, smooth, about 1-2 mm in diameter, creamy colony, circular and non-hemolytic due to the absence of hemolysis enzyme (Paymani *et al.,* 2011).

Biochemical test

The probable *A. baumannii* samples were then put through a series of biochemical assays. All 50 samples tested for catalase were positive and this result agreed with the work Malini *et al.* (2009), who also found a positive catalase result for *A. baumannii* isolates. Oxidase test was negative for all 50 *A. baumannii* isolates which showed no colour change and this result agreed with (Malini *et al.,* 2009), who indicated a negative Oxidase result for *A. baumannii* isolates. This test detects the presence of a cytochrome oxidase system (MacFaddin, 1972). An intracellular oxidase enzyme is produced by animals that possess cytochromes. This oxidase enzyme catalyzes the oxidation of cytochrome c. Oxidase-positive organisms color the solution blue or purple because they contain cytochrome C as a respiratory chain component. Oxidase-negative organisms do not oxidize the substance, leaving it white within the parameters of the test, and are, therefore, cytochrome c deficient (Patricia and Laura, 2010).

All 50 *A. baumannii* isolates had indole-negative test *A. baumannii* did not break the tryptophan in the medium since the indole test checks an organism's capacity to break down the amino acid tryptophan and generate indole (Maria, 2009). Thus no colour change occurred upon the addition of the Kovács reagent. The reagent appeared as a thin yellow layer on top of the culture medium and this agreed with Malini *et al.* (2009), which had a negative indole result for *A. baumannii* isolates. Simmon citrate tests were positive for *A. baumannii* isolates (Sofia *et al.,* 2004). This test evaluates a bacterium isolate's capacity to use citrate as a carbon and energy source (MacFaddin, 2000). The medium's pH was raised to above 7.6 by the alkaline carbonates and bicarbonates created as byproducts of citrate catabolism, which caused the bromothymol blue to change from its initial green color to blue that caused positive outcomes (Maria, 2009).

Vitek-2 System

Vitek-2 compact automated system assays were then used to corroborate the isolates' identity as *A. baumannii* as shown in Table 3, 4). It was regarded as a quick and effective technique for identifying microorganisms. The method included multiple physiological measures for bacterial species determination. The results were obtained after 18 hrs. of incubation in the device with an accuracy of about 99 % (Joyanes *et al.,* 2001).

Antibiotic susceptibility of *A. baumannii*

The high resistance of *A. baumannii* to amoxicillin and vancomycin agreed with Dema *et al.* (2020), who reported that vancomycin resistance rate was 95.8% for *A. baumannii* and with Azizun *et al. (*2013), who reported that *Acinetobacter* isolates from different clinical

Table 6. Antibiotic sensitivity results for *Acinetobacter baumannii* isolates

Class	Antibiotic disc	Resistance	Intermediate	Sensitive
Penicillin	Amoxicillin	49 (98%)	1 (2%)	_
Glycopeptides	Vancomycin	49 (98%)	1 (2%)	_
Pipopeptides	Colistin	48 (96%)	2 (4%)	_
Pipopeptides	Polmyxin	48 (96%)	2 (4 %)	_
Aminoglycosides	Gentamicin	35 (70%)	8 (16%)	7 (14%)
Macrolides	Azithromycin	33 (66%)	13 (26%)	4 (8%)
Monobatams	Aztreanam	19 (38%)	28 (56%)	3 (6%)
Quinolones	Ciprofloxacin	19 (38%)	29 (58%)	2 (4 %)
Aminoglycosides	Amikacin	8 (16%)	32 (64%)	10 (20%)
Quinolones	Levofloxacin	7 (14%)	28 (56%)	15 (30%)

sources were 100% resistant to *amoxicillin. A. baumannii* was associated with a modifying enzyme that modifies antimicrobial molecules. The perfect example of these modifying enzymes is the aminoglycosides modification enzyme (Salimizand, *et al.,* 2018).
While Ilkay *et al.* (2013) reported that the resistance of colistin was 96% and agreed with current results, increasing use of colistin and the presence of multidrug-resistant when treating *A. baumannii* infections that are resistant to carbapenem. Clinicians should use colistin with other antibiotics or beta-lactamase inhibitors to reduce the chance of acquiring resistance to colistin. Besides, the resistance of *A. baumannii* to Gentamicin was 70%. This percentage was consistent with the work of Zainab *et al.* (2018), who demonstrated a close percentage of 66.7% of gentamicin in wound isolate. Nevertheless, this research was inconsistent with Levofloxacin resistance (75%) in sputum isolate. The resistance of *A. baumannii* to azithromycin was moderate (66%). Zainab *et al.* (2018) reported a close result where the Azithromycin resistance rate was 57.1%.
The results of aztreanam show a moderated resistance activity of 38 %, which agreed with Azizun *et al.* (2013), where the percentage was 38 % and with Haider and Jabbar (2020), who demonstrated that the percentage of gentamicin was 30%. Ciprofloxacin resistance was reported with a percentage of 38% for the tested isolates and agreed with Saif and Wathiq (2019), who mentioned a near percentage of 40%, while disagreed with Khalidi *et al.* (2017), who demonstrated that the resistance of Ciprofloxacin was 92%. Levofloxacin resistance was 14% for the tested isolates and was close to the results of Ilkay *et al.* (2013), who reported the resistance of Levofloxacin at 16%, while it disagreed with Arjmand *et al. (*2020), who reported that the *A. baumannii*'s resistance to levofloxacin was 54.5%. The present result was consistent with the result of Safari and Alikhani (2013), who showed that 91% of the isolates were resistant to levofloxacin. Amikacin antibiotic revealed low activity against tested *A. baumannii* isolates with a percentage of 16%. Zeina *et al.* (2022) reported a close result of amikacin sensitivity rate of 22%. The highest resistance rate was observed against Amoxicillin and Vancomycin 98% of the isolates resistant, followed by Colistin and Polmyxin with percentage of 96% resistance, and 70% for gentamicin. The resistance rate against the other antibiotics was 66% against azithromycin, aztreana, ciprofloxacin, 38%, amikacin (16%), and the lowest resistance against levofloxacin was 14%. Previous data from Iraq and Afghanistan showed that some of the most frequent microorganisms causing serious and frequently fatal wound infection are highly resistant forms of *A. baumannii* (Beceiro *et al.,* 2014). These microbes have developed a wide range of antibiotic resistance for various causes. Antibiotic resistance genes frequently promote biofilm development and are one of these most significant factors (Meredith *et al.,* 2017). Additionally, the membrane's decreased permeability is a key factor in the rise in *A. baumannii's* drug tolerance. They believed that particle size reduction led to a rise in antibiotic resistance, particularly for chloramphenicol (Vrancianu *et al.,* 2020).
It had been found that most of the *A. baumannii* isolates were multi-drug resistant (MDR) (98%), while the other isolates (2%) were extensively drug-resistance (XDR) to the majority of antibiotics tested (Fig. 2). This percentage was consistent with the work of Castilho *et al.* (2017), who reported a high percentage (93%) that were also MDR.

Biofilm production profile

The results indicated that from a total number of 50 isolates, biofilm formation was observed in the 50 (100%) isolates. Strong biofilm was detected in 40 (80.0%) of the tested isolates, while 10 (20.0%) of them were able to form moderate biofilms with an OD of 630 nm and mean values of ELISA reader was in the range between (0.1 – 0.2). On the other hand, there were (0.0%) no isolates that were recognized as weak biofilm formation. There were no non-producers isolated, as shown in Table 7.

Molecular identification

Genomic DNA Extraction

All thirty A.baumannii isolates' genomic DNA was effectively recovered using a commercial Genomic DNA purification Kit (Bioneer Company/Korean) in accordance with the manufacturer's directions. The extraction findings were successful, and the Nanodrop spectrophotometer at 260/280 nm was used immediately to quantify the DNA (concentration and purity). All of the samples' extracted DNA concentrations varied from 58 to 141 ng/l. The assessed purity levels of extracted DNA was verified and examined using Gel electrophoresis in 1% agarose for 60 minutes at 75 volts. The DNA is visible as dense lines, as shown in Fig. 3.

Molecular detection of *A. baumannii* species by 16SrRNA gene

All of the isolates were properly identified by a particular primer for the 16S rRNA gene, which was used to carry out a one-step conventional PCR method. The 16srRNA gene was detected in the all A. baumannii isolates, after extracted the DNA, and a PCR product size single amplicon 1494 bp is shown in Fig. 4, indicating that all clinical isolates were of *A. baumannii.* The results of the PCR products were confirmed by comparing their molecular weight with 1500 bp DNA Ladder.

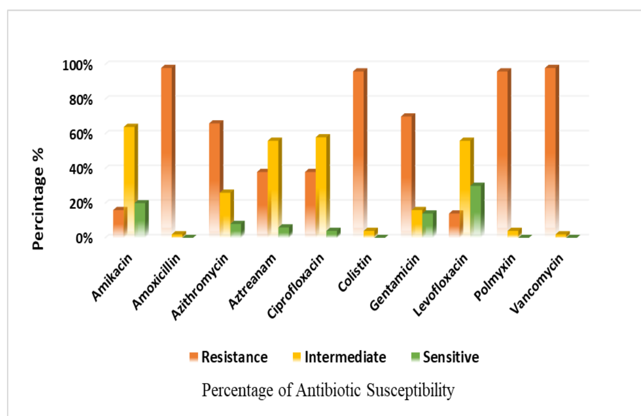

Fig. 2. *Distribution of Antibiotic sensitivity test of Acinetobacter baumannii*

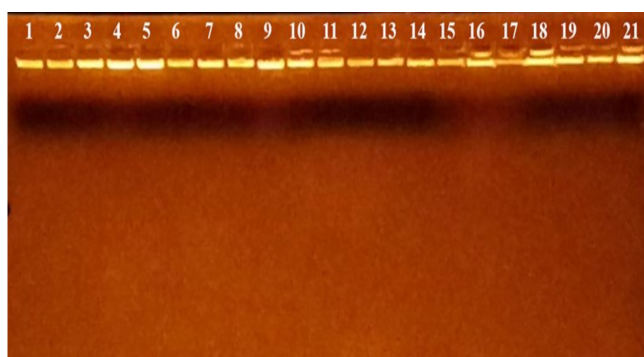

Fig. 3. *Genomic DNA bands from amplified A. baumannii strains stained with ethidium bromide after being separated on (0.7%) agarose, 75V, for 60 minutes.*

Table 7. Classification of bacterial isolates according to OD of NC in micro-titer plate assay method

Mean OD value of NC	Adherence Biofilm Formation
0.05< ODA < 0.1	Non adherent / Weakly adherent
0.1 < ODA < 0.2	Moderately adherent
0.2 < ODA	Strongly adherent

OD- Optical density; ODA- Mean of three optical densities of each isolate; NC- Negative control

Table 8. Biofilm-forming ability of *Acinetobacter baumannii* by using micro-titer plate assay

Type of biofilm formation	No. (%)	Probability
Strong	40 (80.0%)	
Moderate	10 (20.0%)	0.000041
Weak	0 (0.0%)	
Total	50 (100.0%)	

The present study's results agreed with Urdan (2005), who reported the genetic identity of an *Acinetobacter* clinical strain with a high rate of genomic identification. They used the gene 16S rRNA. Additionally, there is consensus in other studies that identifying and sequencing this gene is reliable for identifying clinical samples of *Acinetobacter* (Babapour *et al.,* 2016).

Conclusion

The present study concluded that most of the *A. baumannii* isolates (98%) were multi-drug resistant (MDR) and all fifty isolates could form a biofilm. Most of them (80%) were strong biofilm forming. The other 20% were moderate. The results showed a higher association of *A. baumannii* with high antibiotic resistance. The highest resistance rate was observed against amoxicillin and vancomycin, with 98% of the isolates resistant, followed by colistin and polmyxin. In contrast, the lowest resistance was observed against levofloxacin which

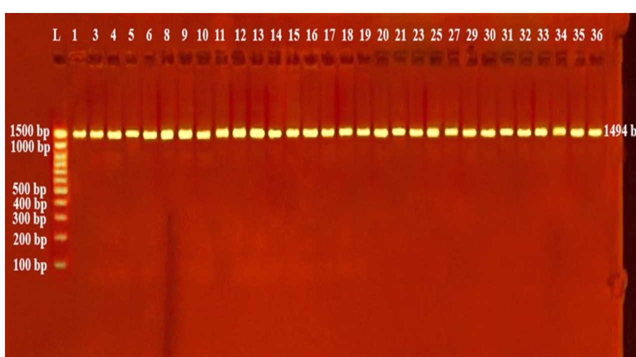

Fig. 4. *16SrRNA bands from A. baumannii strains, amplified on 2 % agarose at 75 volts for 60 min and labelled with ethidium bromide*

showed moderate sensitivity against MDR *A. baumannii*.

Conflict of interest

The authors declare that they have no conflict of interest.

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