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Molecular Diversity and Biofilm Formation in Extensively Drug-Resistant *Acinetobacter baumannii* Isolated from Patients in Diyala

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ABSTRACT

Acinetobacter baumannii is a worldwide threat that causes infections in hospital settings because of multidrug resistance to widely available antimicrobial drugs. The study aimed to determine antimicrobial susceptibility and biofilm formation in *A. baumannii* clinical isolates and the molecular diversity of these isolates by using *16S rRNA* Gene. The bacterial isolates have been recovered from patients admitted to Baquba Teaching Hospital Diyala, Iraq. Diagnoses were then confirmed using biochemical tests, VITEK-2 system and molecular screening of 16S rRNA Gene were done by PCR. 30 distinct isolates were identified as *A. baumannii* throughout the collection. All *A. baumannii* isolates were resistance to (Piperacillin, Ceftazidime, Cefotaxime, Ceftriaxone, Piperacillin–tazobactam, Ticarcillin–clavulanic acid, Gentamicin) in percentage (100%) While resistance to Amikacin 96.7%, Imepenem and Meropenem 76.7%, Levofloxacin 66.7% and finally to Ciprofloxacin 63.3%, 11isolates (36.7%) were MDR, and 19 (63.3%) isolates were XDR. The results of this study also obsrved there were 19 moderate biofilm producing strains and 8 isolates which weak, While only 3 isolates stong biofilm. The results showed 13 A.bumaannii isolates 43.3% were given positive results for metallo beta-lactamase versus 17 isolates 56.7% given negative results. A total of eight samples (assigned S1 - S8) were included in the present study. The conducted sequencing reactions of 16SrRNA amplicons indicated the accurate identity of the investigated samples, which were found to be attributed to *Acinetobacter baumannii*. The alignment of nucleic acid sequences of the eight isolates revealed the presence of four nucleic acid variants distributed in S1 – S8 samples. Unique accession numbers were obtained for all analyzed sequences and each sequence took accession number., 16S rRNA sequencing is an effective approach for identifying and characterizing clinical samples of *Acinetobacter baumannii*, with various clinical benefits such as high sensitivity and specificity, cheap cost, and quick turnaround time.

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1. INTRODUCTION

Acinetobacter baumannii is an opportunistic gram-negative bacteria that ranks among the top seven reasons of concern in human health care [1]. It is the leading cause of nosocomial infections such as pneumonia, meningitis, urinary tract infections, and respiratory infections. This bacterium can also be identified in wound infections like burns and in patients admitted to intensive care units (ICUs) [2]. The most prevalent and dangerous multidrug-resistant (MDR) pathogens, as determined by the World Health Organization (WHO), are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, Pseudomonas aeruginosa, and Enterobacter spp. These pathogens are collectively referred to as ESKAPE [3]. Its exceptional plasticity and propensity to mutate in reaction to its surroundings challenge to researchers. Its ability to endure in clinical environments raises the risk of infection and transmission [4]. *A. baumannii* is virulent due to a number of variables, including phospholipase, lipopolysaccharide, capsules, outer membrane proteins, efflux pumps, quorum sensing, protein secretion systems, and nutrient acquisition systems. These elements aid the pathogen's development of antibiotic resistance and ability to endure harsh environments. [5]. Bacterial colonies that are tightly adhered to a surface or to one another form biofilms, which are encased in a matrix made up of proteins, exopolysaccharides (EPS), extracellular DNA, and water channels [6]. Biofilm-forming bacteria are highly resistant to antibiotics, anti-infectives, and human white blood cells [7].

Acinetobacter baumannii, which may form biofilms, is highly resistant to desiccation, nutritional restriction, and antibiotic therapies, making it a considerable issue for medical personnel [8]. *A.baumannii* isolates have developed multiple mechanisms of antibiotic resistance, including changes to the target sites, increased expression of efflux pump-associated genes, reduced permeability, alterations to penicillin-binding proteins (PBP), and enzymatic deactivation of drugs, such as beta-lactamases. Among these mechanisms, the inactivation of beta-lactam antibiotics through beta-lactamase enzymes is considered one of the most significant forms of resistance in A. baumannii [9].The β-lactamases are divided into four groups as class A, class B (metallo β-lactamases), class C and class D (oxacillinases) [10]. The resistance of A. baumannii to carbapenems referred explicitly to as carbapenem-resistant *A. baumannii* (CRAB), can confer a high resistance level. In addition to the intrinsic resistance determinants, oxacillinases belonging to Ambler Class D and Metallo β-lactamases classified under Ambler Class B play significant roles in conferring resistance to carbapenems [11]. The 16S rRNA gene constitutes a vital component of the ribosome's structural RNA elements. Prokaryotes possess 5S and 23S ribosomal RNA (rRNA) species within their big subunits, with 16S rRNA species within their small subunit. The proposal suggests that the 16S-23S internal transcribed spacer plays a significant role in detecting bacterial identification at the species level. Sequence analysis investigations have demonstrated the utility of 16SrRNA in the genus-level title of Acinetobacter [12]. Based on the given information, this study aimed to determine antimicrobial susceptibility and biofilm formation in *A. baumannii* clinical isolates and the molecular diversity of these isolates by using the 16S rRNA Gene.

2. METHOD

2.1.Isolation and Identification

The bacterial isolates were obtained from patients admitted to Baquba Teaching Hospital and Al-Batool teaching hospital in Diyala, Iraq. Total of 200 clinical samples in this study were collected from various sources, such as wounds, burns, sputum, and urine from October 2022 to March 2023. A total of 30 isolates were subjected to isolation and purification using standard bacteriological culturing techniques on blood agar, MacConkey agar medium, and HiCrome™ Acinetobacter Agar Base. The isolates were confirmed using the Vitek 2 compact system manufactured by Biomérieux [13].

2.2. Antibiotic sensetivity test (AST) of *A.baumannii*

An estimation was conducted to assess the possible resistance of *A. baumannii* isolates against 14 antibiotics belonging to various classes. To evaluate the potential resistance of *A. baumannii* isolates against a range of antibiotics from different types, 30 isolates were subjected to an antibiogram test following the guidelines set by the Clinical and Laboratory Standards Institute [14]. The Kirby-Bauer disk diffusion technique was employed for this assay, utilizing disks obtained from Bioanalyse, Turkey. The antibiotics tested included Piperacillin, Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone, Imipenem, Meropenem, ampicillin-sulbactam, piperacillin-tazobactam, ticarcillin-clavulanic acid, Gentamicin, Amikacin, Ciprofloxacin, and Levofloxacin. The antibiogram test was performed on Mueller Hinton agar. The colonies of inoculum were generated overnight, and a culture of The isolates was introduced into a 3ml tube containing normal saline. Subsequently, the turbidity was compared to a level of 0.5 McFarland. the isolate was categorized into three groups based on its susceptibility to antibiotics: sensitive, moderate, or resistant [14].

2.3. Biofilm Form ation Assay

2.3.1 Congored agar (CRA) (Qualitative assay)

To qualitatively assess biofilm production, the Congo Red method was conducted according to the procedure described by Freeman et al. [15]. A concentrated aqueous solution of Congo Red stain was prepared by dissolving 0.8 g of the stain in 200 ml of distilled water. This solution was prepared separately from the other medium elements and subjected to autoclaving. The dye exhibits interactions with specific polysaccharides in the biofilm, resulting in complexes with distinct colors. A solution was prepared by dissolving 37 g of brain heart infusion agar and 50 g of sucrose in 800 ml of distilled water, followed by autoclaving. Subsequently, the agar was cooled to a temperature of 55˚C, following which the Congo Red stain (200 ml) was introduced. The bacterial isolates were presented onto the media prepared in advance and placed in an aerobic environment at 37°C for 24 hours. Black colonies exhibiting a dry crystalline texture indicated a robust biofilm production. The colonies that did not generate biofilms were identified as red, with sporadic darkening observed at the central region of the colonies.

2.3.2 Microtiter Culture Plate Method (MTP) (Quantitve assay)

To quantitatively determine biofilm formation, a microtiter plate colourimetric assay was conducted according to Jaffar et al. [16]. The procedure involved the following steps: All isolates were grown in brain heart infusion broth for 24 hours at 37°C, and then the turbidity was adjusted to McFarland 0.5 by transferring 100 µl of bacterial growth into a 2 ml tube of normal saline.Sterile flat-bottomed 96-well polystyrene microtiter plates were filled with 180 µl of brain heart infusion broth containing 1% glucose.Then 20 µl of bacterial suspension from normal saline was added to three wells of the microtiter plates, while six wells containing bacteria-free broth were used as negative controls. The plates were covered and incubated aerobically without shaking for 24 hours at 37°C, followed by gentle washing with PBS and air-drying.

To fix the biofilms, 150 µl of methanol was added to each well for 15 minutes at room temperature, followed by washing and air-drying.The plates were stained with 250 µl of 1% crystal violet solution for 15 minutes at room temperature. After washing and drying at 37°C for 15 minutes, the wells were checked for complete dryness.The dye was resolubilized by adding 200 µl of 95% ethanol to each well for 30 minutes. The optical density (OD) of each well was read at 630 nm using a microtiter plate reader. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Based on the ODc value, all isolates were classified into four groups: non-producer, weak biofilm producer, moderate biofilm producer, and strong biofilm producer (Table1) [17].

Table (1): The Biofilm intensity.

Steps	°C	m: sec	Cycle
1. Initial Denaturation	94	05:00	1cycle
2. Denaturation	94	00:45	
3. Annealing	56	01:00	35cycle
4. Extension-1	72	01:00	
5. Extension -2	72	07:00	1cycle

*Cut off value (ODc)= average OD of negative control + (3*Standard Deviations)

Table 2. The specific primers' pair selected to amplify 16S rRNA within A. baumannii genomic DNA sequences.

Mean OD	Biofilm intensity
OD <= ODc*	Non – adherent
2ODc> OD> ODc	Weak
4ODc> OD> 2ODc	Moderate
OD > 4 ODc	Strong

2.4 Detection of Metallo-beta-lactamase production by combined EDTA disc test (CEDT)

To detect Metallo-beta-lactamase (MβLs) production, *A. baumannii* isolates were suspended in normal saline after 24 hours of incubation, equivalent to 0.5 McFarland. Next, 0.1 ml of the suspension was spread on Muller Hinton agar plates using a sterile cotton swab and left to dry at room temperature. Two Imipenem (10µg) discs were placed 20 mm apart on the plate, and 5 µl of EDTA was added to one of the Imipenem discs. The plate was then incubated overnight at 37°C. If an enhanced zone of inhibition (about 7mm or more) around the Imipenem + EDTA disc was observed compared to the disc without EDTA, it suggested a positive result for MβLs production [18]. This detection method is known as the combined EDTA disc test (CEDT).

2.5. Molecular detection

DNA of eight Extensively Drug Resistant *Acinetobacter baumannii* in were extracted by ZR Fungul/Yeast/Bacterial DNA MiniPrep according to the manufacturer company instruction (ZYMO) from the USA. After measurement the concentration and purity of the DNA were mixed with primer as in (Table2) [19]. Genomic DNA of *Acinetobacter baumannii* was used to detect the 16SrRNA genes and that has been performed through the conventional PCR with 25µl of the PCR Master mix reaction.

Table 3. Thermal cycling condition used for 16S rRNA primers DNA

Primer	Sequence (5′-3′)	Amplicon size	Annealing temperature	Ref.
27-F	AGAGTTTGATCCTGGCTCAG	1500 bp	56°C	19
1492R	TACGGTTACCTTGTTACGACTT			

The program that used in the thermocycler PCR was carried out in with annealing at 56°C for 1 min. The primer were provided by (Integrated DNA Technologies /USA) showen in Table 3. The amplified PCR products were detected by using agarose gel electrophoresis.

2.6.Sequencing of PCR amplicons 16S rRNA

Eight XDR isolates (designated S1 - S8) were included in the current investigation, two from each wound, burn, urin, and sputum were chosen for sequencing. PCR products were transferred to Macrogen Corporation in Korea for Sanger sequencing utilizing an automated DNA sequencer called the ABI3730XL. Using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA), the sequencing results of the PCR products of the selected bacterial sample were edited, aligned, and assessed along with the corresponding sequences in the reference database. Each sequenced sample's detected changes were given a number in both its matching position in the reference genome and its PCR amplicon.

2.7.Deposition of sequences to GenBank

All the investigated and analyzed sequences were submitted to the NCBI Bankit portal and all the instructions described by the portal were followed as described by the server [20]. The submitted sequence was provided as nucleic acid sequences in the NCBI to get a unique GenBank accession number for the investigated sequences.

2.8. Comprehensive phylogenetic tree construction

In this work, a precise, comprehensive bacterial tree was created by the methodology outlined by Sarhan [21]. Using the NCBI-BLASTn server, the detected bacterial variations were compared to their nearby homologous reference sequences [22].Then, a comprehensive tree encompassing all relevant variants was constructed using the neighbor-joining approach. The resulting tree was then displayed as a polar cladogram using the Figtree program (http://tree.bio.ed.ac.uk/software/figtree/). The comprehensive tree was color-coded to distinguish the sequences of each included species from one another. In contrast, the color assigned to each clade was treated as a singular, cohesive hue.

Ethical Clearance: The samples were gained according to Local Research Ethics Committee approval in the College of Science, University of Diyala, No. 25-ASJ-Sci 168 in 1/12/2025.

Statistical analysis

The SPSS v.23 program was used to analyze current data. Categorical variables were compared using Fisher's exact test or Pearson's chi-square test to compared between percentages. A level of significance of P=0.05 was applied to test.

3. RESULTS AND DISCUSSION

Two hundred clinical samples were examined, from which 30 clinical isolates of gram-negative bacteria were obtained. These isolates were primarily recognized as *A. baumannii* and were gathered from various clinical sources. The origin of these isolates can be described as follows: 12(40.0%) isolates from Wounds, 8(26.7%) isolates from Burns, 6(20 %) isolates from sputum, and the last 4 (13.3%) isolates from urine (Table 4). These differences statistically were non-significant (Chi-square=10.07, *P*-value=0.12).

Table 4. Distribution the diagnosis of bacteria from clinical samples (n=200)

Clinical samples	Diagnosis of bacteria			Total	P-value
	No growth	Other species	*A.baumannii*		
Woundswabs	5 (14.7%)	33 (24.3%)	12 (40.0%)	50 (25.0%)	
Burn	13 (38.2)%	29 (21.3%)	8 (26.7%)	50 (25.0%)	Chi-square=10.07
Urine	9 (26.5%)	37 (27.2%)	4 (13.3%)	50 (25.0%)	P-value=0.12
Sputum	7 (20.6%)	37 (27.2%)	6 (20.0%)	50 (25.0%)	(N.S)
Total	34 (100.0%)	136 (100.0%)	30 (100.0%)	200 (100.0%)	

3.2 Antibiotic Susceptibility test

This investigation discovered that *A. baumannii* clinical isolates were highly resistant to the majority of the antibiotics tested (Figure1). *A. baumannii* isolates were resistance to (Piperacillin, Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone, Ampicillin–sulbactam, Piperacillin–tazobactam, Ticarcillin–clavulanic acid, Gentamicin) in percentage (100%) While resistance to Amikacin 96.7% Imepenem and Meropenem 76.7%, Levofloxacin 66.7% and finally to Ciprofloxacin 63.3% . Results conducted in the current study agree with Iraqi study in Al-Najaf City [23] which reported that *A. baumannii* isolates resistance 100% to Cephalosporins. The current results disagree with other local Iraqian study in Erbil hospitals [24] that shows resistance in percentage 86% for Ceftazidime and 4% for Ceftriaxone and Cefepime. The current study showed that A.bumannii isolates displayed a high degree of antibiotic resistance, resistant to at least one antibiotic in three or more of the ten antimicrobial classes assessed in this investigation. According to the specified criterion, multi-resistant isolates were categorized into three groups: MDR, likely XDR, or possible PDR. Of 30 isolates, 11 (36.7%) were classified as MDR, while 19 (63.3%) were categorized as XDR. The epidemiological importance of extensively drug-resistant (XDR) bacteria lies in their ability to resist several antimicrobial medications and their distinct inclination to acquire resistance against nearly all or a majority of antimicrobial treatments [25].

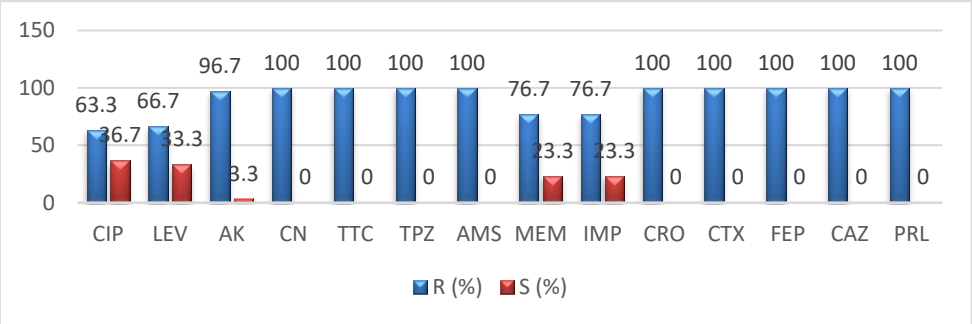


Figure1: Antibiotic Susceptibility profile of *Acinetobacter baumannii* (n=30).

Abbreviations: CIP: Ciprofoloxacin; LVE: Levofloxacin; AK: amikacin; CN: Gentamicin; TTC: Ticarcillin –clavulanic acid; TZP: Piperacillin -tazobactam; AMS: Ampicillin-sulbactam; MEM: meropenem; IPM: imipenem; CRO: Ceftriaxone; CTX: Cefotaxime; FEP: Cefepime; CAZ: ceftazidime;PRL: Piperacillin.

3.3 Detection of the Bacterial Ability for Adherence and Biofilm Formation:

This study assessed the adhesion ability and slime layer production (biofilm formation) of 30 *A. baumannii* isolates. Two traditional phenotypic methods, Congo-red agar (CRA) and Microtiter plate (MTP), were employed. The results were measured using an ELISA reader, as depicted in Figure2.

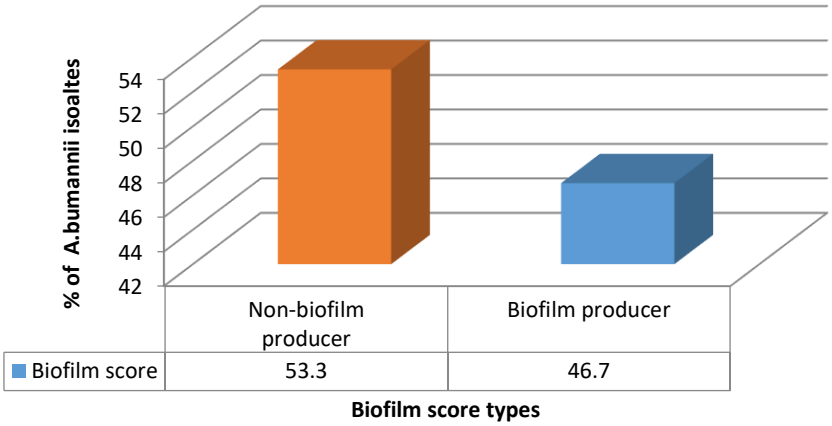


Figure2. Distribution of the *A.bumannii* isolates (n=30) according to biofilm-producing scores with the congo red method.

The results of this study observed there 16 /30 *A.bumaannii* isolates scored as non-biofilm producers by the congo red method and had 0.58 concentration when compared with microtiter plate method, while the remaing isolates 14/30 scored as biofilm prodcuer and had 0.61 concentration when comapred with microtiter plate method,These differences statistcally were non-significant (P-value=0.66). The results of this study also observed there were 19/30 had 0.56 biofilm concentration (O.D) which scored as moderate biofilm producing strains and 8/30 isolates had 0.5 (O.D) which scored as weak biofilm producing strains, while there were 3/30 isolated had 1.04 (O.D) which scored as strong biofilm producing strains, These differences had P-value ≤0.001 (Table5).

Table5. Comparision between the congo red and microtiter plate method for categorized the *A.bumaannii* for biofilm production (n=30)

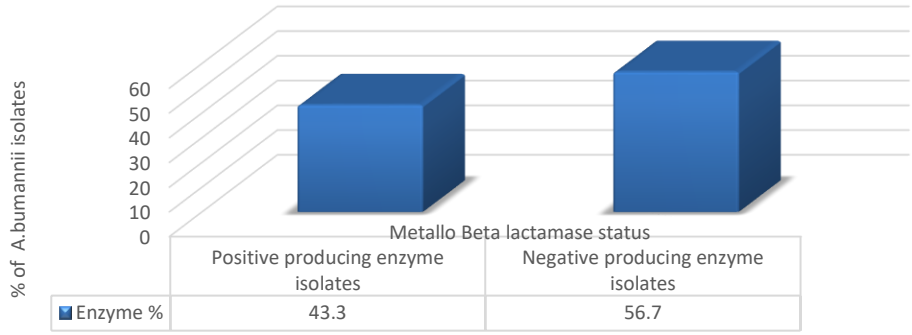
Biofilm method	Score	N	O.D value		P-value
			Mean	SD	
Congo red	Non-biofilm producer	16	0.58	0.17	0.66 (N.S)
	Biofilm producer	14	0.61	0.21	
Plate method	Weak	8	0.5	0.07	≤0.001 H.S
	Moderate	19	0.56	0.11	
	Strong	3	1.04	0.01	

The congo red agar method (CRA) is not advised to be used as a routine approach for the development of biofilm because it can generate confusing results and has a low detection sensitivity [26]. Microtiter plate assay is a significant approach for the research of the early stages of biofilm adhesion, and it allows for the production of a biofilm on both the wall and the bottom of the microtiter plate. Because it enables more accurate, and rapid to quantify contact cell attachment and biofilm formation of various bacterial strains, it has been the most popular method for detecting the production of biofilms and has been regarded as the gold standard for this purpose. In spite of the fact that the adhesion detection utilized by microtiter plate assay is sensitive to a wide range of situations, this method is still superior to Congo red agar and in terms of its accuracy when applied to biofilm detection [27].

3.4 Phenotypic Detection of Metallo-beta-lactamase production by combined EDTA disc test (CEDT)

The results of this study observed there 13 *A.bumaannii* isolates 43.3% were given positive results for metallo beta-lactamase versus 17 *A.bumaannii* isolates 56.7% were given negative results for metallo beta-lactamase as shown in Figure 3.

The result in the current study shows nearly to result in Nepal that show *A. baumannii* comprised 36.4% producing metallo beta lactamase enzyme [28]. *A. baumannii* exhibits resistance to β-lactam antibiotics by producing β-lactamases, enzymatic proteins that effectively degrade these valuable therapeutic drugs. Throughout history, the carbapenem family of β-lactam antibiotics has been widely regarded as the preferred pharmacological intervention for treating *A. baumannii* infections. Nevertheless, the efficacy of these treatments has been considerably reduced due to the pathogen's ability to produce carbapenem-hydrolyzing class D β-lactamases (CHDLs) [29].



Fiqure 3. Distribution the *A.bumannii* isolates (n=30) according to Metallo Beta lactamase enzyme production

3.5 Sequencing and phylogeny analysis of 16S rRNA gene

A total of eight samples (assigned S1 - S8) were included in the present study. All of the amplified amplicons required to exhibit distinct, clear, and crisp bands before the 16S rRNA amplicons were sent for sequencing. The conducted sequencing reactions indicated the accurate identity of the investigated samples, which were found to be attributed to *Acinetobacter baumannii*. The alignment of nucleic acid sequences of the eight isolates with the reference sequences of *Acinetobacter baumannii* (GenBank acc. no. NZ_CP043953.1) revealed the presence of four nucleic acid variants distributed in S1 – S8 samples. 81C>T was detected in S1-S3,S5-S8 samples, while 197C>T was identified in all samples (Table 6 and Figure4). Whereas 446C>T and 461G>A were both identified in S1, S2, S4, S5, and S6 samples. It was inferred from the tree that the investigated samples occupied two distinct phylogenetic positions and they suited in the vicinity of one clinical strain that was deposited from the USA. It was inferred from the tree that the investigated samples were derived from the other incorporated strains within the same clade. Accordingly, all the observed variants exerted a noticeable effect on changing the phylogenetic positioning of the investigated samples within the incorporated clades of *Acinetobacter baumannii*.

Table 6. The pattern of the observed mutation in the PCR products of the 16S rRNA amplicons of *A. baumannii* in comparison with its corresponding NCBI referring sequences (GenBank acc. no. NZ_CP043953.1). The letter "g" refers to "genomic".

No.	Sample	Native	Allele	Position in PCR fragment	Position in the reference genome	Variant summary
1.	S1-S3,S5-S8	C	T	81	18245	g.18245C>T (81C>T)
2.	S1-S8	T	C	197	18362	g.18362T>C (197T>C)
3.	S1,S2,S4,S5,S6	C	T	446	18611	g.18611C>T (446C>T)
4.	S1,S2,S4,S5,S6	G	A	461	18626	g.18626G>A (461G>A)

16S rRNA sequencing is a widely used molecular technique for identifying *A.baumannii* at the species level, based on the sequence variations in the 16S rRNA gene, which is present in all bacterial genomes. Many studies have used 16S rRNA sequencing to detect the presence of *A.baumannii* in clinical samples and to discover genetic variants within these organisms. For example, one study used rRNA sequencing to identify and characterize *Acinetobacter baumannii* isolates from clinical samples. The researchers discovered that 16S rRNA sequencing may distinguish between distinct subtypes of *Acinetobacter baumannii* based on changes in the gene sequence. The study also discovered that *Acinetobacter baumannii* isolates from various clinical sources exhibited a high genetic diversity, as well as distinct subgroups [30].

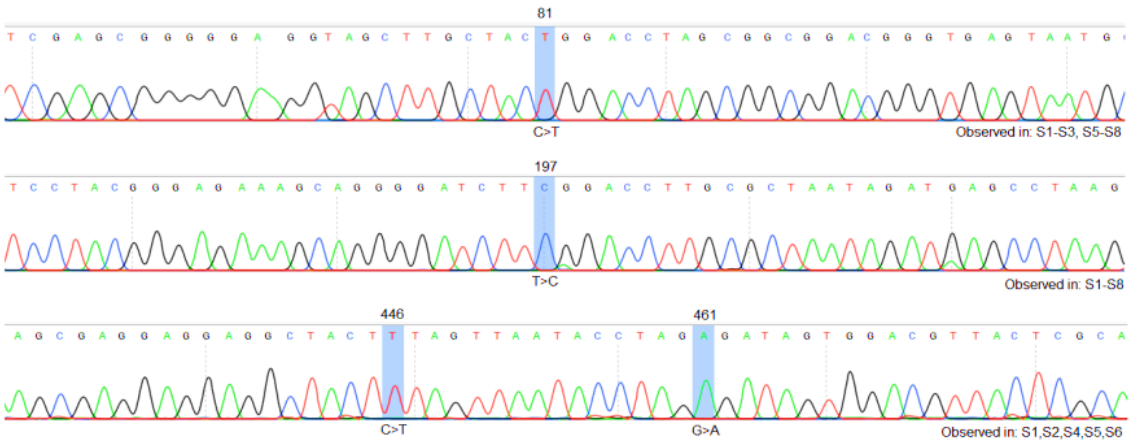


Figure 4. The chromatogram was obtained by analyzing the bacterial samples of *A.baumannii* under inquiry. The symbol "S" is the code allocated to the models being investigated in this study. The highlighted sections correspond to the sites where nucleic acid changes have been detected.

Eight strains of *A. baumannii* were isolated from human sources in Baquba/Diyala. The 16srRNA gene sequences of these strains were later submitted to GenBank, a well-known genetic sequence database. Experts from GenBank thoroughly analyzed and examined the obtained sequences. The strains were published in the National Center for Biotechnology Information (NCBI), and their database entry was recorded in the DNA Data Bank of Japan (DDBJ). All sequences have been approved for the gene bank and given accession numbers (MK503659, MK559695). Table 6 shows the specific locations of the discovered variants, which provide a detailed summary of the findings resulting from the sequenced 1498 base pair fragments. The ribosomal sequences used in this work were archived on the NCBI online server. Each sequence has been issued a unique accession number. The sequences investigated in this work were assigned the accession numbers OQ996976, OQ996977, OQ996978, OQ996979, OQ996980, OQ996981, OQ996982, and OQ996983. These accession numbers correspond to the S1, S2, S3, S4, S5, S6, S7, and S8 samples, respectively.

To provide a phylogenetic understanding of the actual distances between the four investigated bacterial samples obtained from four different clinical sources, a comprehensive phylogenetic tree was generated in the present study using nucleic acid sequences found in the amplified PCR products of the ribosomal amplicons of *Acinetobacter baumannii* (Figure 5 and Figure 6).

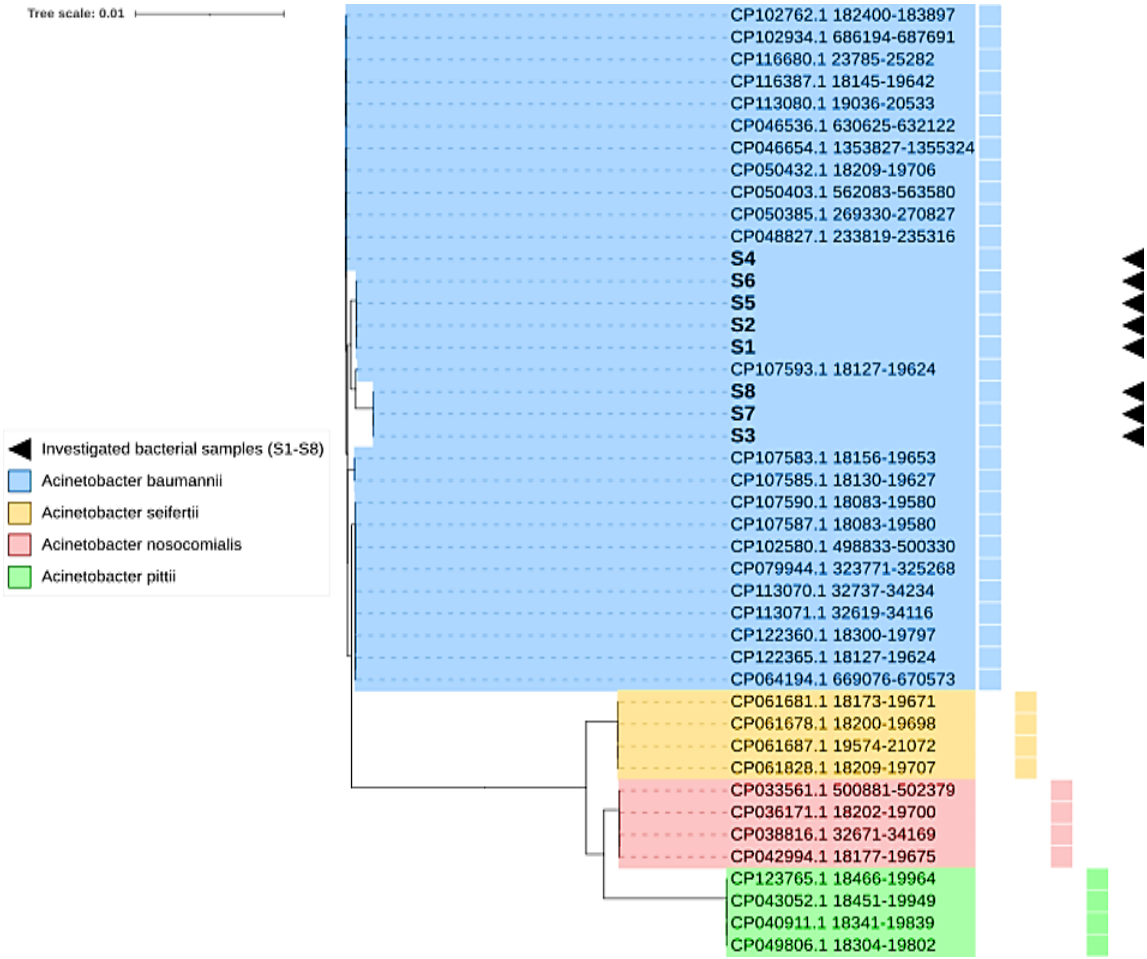


Figure 5. The comprehensive rectangular cladogram phylogenetic tree of the 16S ribosomal fragments for eight samples of *A. baumannii*. The triangular shape of a black color is indicative of the bacterial sequences that have been subjected to analysis. The numbers provided correspond to the GenBank accession number associated with each species described. The numerical values located in the uppermost section of the tree correspond to the extent of variation in scale among the species classified within the all-encompassing tree structure. The letter "S" denotes the code assigned to the samples under investigation.

The observed differences in the distribution of nucleic acid variations may also be associated with the clinical source from which each sample was isolated. This is due to the isolation of S1 - S8 samples from variable clinical sources. Noteworthy, the referred American strain that occupied the nearest phylogenetic position to our samples was a clinically isolated strain. This could partially explain the possible role of the isolation source in the phylogenetic distributions of our samples.

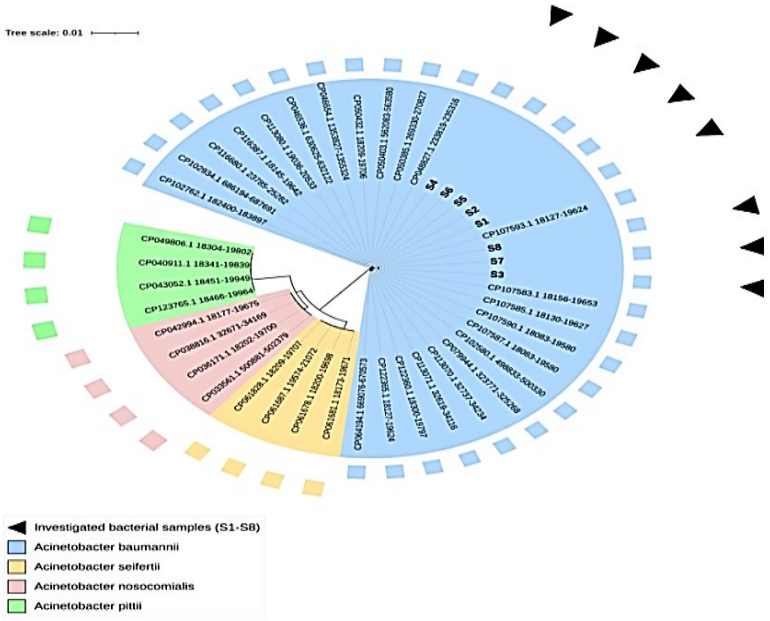


Figure 6. The comprehensive circular cladogram phylogenetic tree of the 16S ribosomal fragments for eight samples of *Acinetobacter baumannii*. The black-colored triangle refers to the analyzed bacterial sequences. All the mentioned numbers referred to the GenBank accession number of each referring species. The numbers at the top portion of the tree refer to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S#" refers to the code of the investigated samples.

4. CONCLUSION

This study found a high rate of extensively drug resistant *A.baumannii* (XDR) isolation with formation of biofilm. 16S rRNA sequencing is a powerful tool for identifying and characterizing clinical samples of *Acinetobacter baumannii* and has several clinical advantages, including its high sensitivity and specificity, low cost, and rapid turnaround time. Therefore, although 16S rRNA sequencing is fundamental for bacterial taxonomy and clinical diagnostics, it should be supplemented with additional molecular targets or whole genome sequencing in order to obtain reliable species-level resolution. These strategies will, when integrated, improve the precision of diagnoses and the identification of AMR, bolster epidemiological surveillance, enhance infection control measures regarding multidrug-resistant pathogens, and aid in managing infections.

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