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Molecular Identification of Virulence Factors and Genotyping of *Acinetobacter baumannii* Isolated from Clinical Samples by ERIC-PCR

Narjis M. Abdulhussein, Mayaada, S. Mahdi

College of Biotechnology, University of AL-Nahrain

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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 "common" virulence factors, it is infections have spread rapidly through hospitals across the globe. The aim of the study 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 during the period from December 2021 to March 2022. Bacterial isolates were identified and the Identification was confirmed by using Vitek-2 system. The result revealed that only (33.3%) isolates were given identical morphological belong to *A. baumannii* isolates. In all fifty clinical isolates of *A. baumannii*, a biofilm production profile was completed. The results showed that in 40 (80.0%) of the tested isolates strong biofilm was detected, while 10 (20.0%) of them were able to form moderate biofilms with an OD of 630 nm and mean ELISA reader values were between (0.1 and 0. 2). According to biofilm production tests, thirty bacterial isolates were selected, which are found to be strong biofilm production. The thirty selected isolates were introduced to molecular assay. The results revealed that all selected isolates (100%) have virulence genes (*CsuE1* and *OmpA* genes). Depending on the thirty isolates, the ERIC-PCR banding patterns have indicated 2 to 6 bands with a total similarity level of about (74.1%). It was concluded.

Keywords: *Acinetobacter baumannii*, Biofilm, *CsuE1* gene, *OmpA* genes, ERIC-PCR.

Corresponding author: (Email: narjis95muthana@gmail.com).

Introduction

The prevalence of infections caused by the Gram-negative opportunistic bacterium *Acinetobacter baumannii* has significantly increased in recent years. The processes driving the success of this pathogen, with only a few numbers of "traditional" virulence factors, continue to be of significant interest (1). *A. baumannii* is capable of causing severe, invasive (usually nosocomial), and highly lethal infections. In recent years, this pathogen has shown signs of multidrug resistance (MDR) (2). Due to the fast expansion of medical device-associated infections and antibiotic resistance, *A. baumannii* biofilms have emerged as one of the most significant global concerns (3). For patients who require mechanical ventilation, *A. baumannii* strains that have the ability to build biofilms on the surface of the endotracheal tube could be extremely dangerous. As a result, this may cause the lower respiratory tract to get colonized at rather high levels (4). A crucial component of Gram-negative bacteria's outer membrane proteins (OMPs), outer membrane protein A (*OmpA*) is a significant virulence factor that regulates the development of bacterial biofilms, eukaryotic cell infection, antibiotic resistance, and immunomodulation (5). By maintaining the mature biofilm on abiotic or biotic surfaces, bap proteins, which are found on the surface of bacterial cells, aid in the establishment and maturation of biofilms. Metal cations and other environmental cues regulate the development of biofilms, which

enhance *A. baumannii's* capacity to stick to specific surfaces (6). Pili, which facilitate adhesion and biofilm formation, are also a major factor in *A. baumannii's* capacity to produce biofilms. Chaperone-usher (CU) route is used to develop the four protein subunits *CsuA/B, CsuA, CsuB, and CsuE* (6) into the Csu pilus. The alternative CU family and the largest family of CU systems, archaic CU pili, combined make up the "nonclassical" branch of the CU superfamily (7). Pili, which mediate adhesion and biofilm formation, are also a major factor in *A. baumannii's* capacity to produce biofilms. *A. baumannii* produces the genes from a Csu operon, which are grouped together to create a pilus-like bundle structure. Consequently, the *CsuE* gene is crucial for the development of *A. baumannii* biofilms. It has been hypothesized that the production of bacterial and fungal biofilms reduces the diffusion of medicines through bacterial and fungal cells and contributes to the persistence of clinical isolates in challenging conditions with multidrug resistance (3).

Identification by PCR is a straightforward and practical molecular method for identifying *Acinetobacter baumannii* isolates (8). In other words, it is essential to examine the isolates at the subtype level in order to distinguish the linked *Acinetobacter* strains that are not interdependent. This objective has inspired the design of some type techniques. On phenotypic tests and molecular methods, various typing systems are developed. Examples of phenotypic typing techniques include biotyping, serotyping, and bacteriocin typing. Enterobacterial repetitive intragenic consensus-polymerase chain reaction (ERIC-PCR) has mostly taken their position (9). The ERIC method commonly considered to be reliable and sensitive, is a much simpler procedure than other methods (10). It is crucial to quickly and accurately identify and genotype *A. baumannii*, especially in burn hospitals, in order to stop the spread of associated nosocomial infections and advance epidemiological research (11).

The aim of the study to revealed genotyping of *Acinetobacter baumannii* isolated from clinical samples and studying its relationship with biofilm formation.

Materials and methods

Sample collection

This study was conducted during the period from December 2021 till March 2022. A total of 150 clinical samples were collected from (respiratory tract secretion (sputum), burns, wounds swaps and urine) were gathered from patients from Ghazi AL Hariri Hospital, Baghdad Teaching Laboratories in Medical City in Baghdad/ Iraq.

Identification of bacteria isolates

All clinical samples were cultured on CHROMagar, MacConkey agar, and blood agar and incubated at 37°C for 24 hr. Each bacterial isolate was identified using morphological, microscopic, and biochemical tests including Oxidase, Catalase, Indol, and Simmon Citrate tests. The Vitek-2 system was used for accurate identification of each bacterial isolate with a 99% confidence level. Finally, the isolates were confirmed through molecular identification by using the 16srRNA gene.

Biofilm formation assay

The ability of *Acinetobacter baumannii* to form biofilms was evaluated using the micro-titer plate assay method (96 wells), as described by Almeida *et al*. (12). This method quantified the biofilm formation of the bacterial isolates as mentioned by Almeida *et al* (12). The classification in the following table (1), which is based on OD values obtained for individual isolates of *A. baumannii*, was utilized for data calculation.

Table (1): Classification of bacterial isolates according to OD of NC in micro-titer plate assay.

Mean OD value of NC	Adherence Biofilm Formation
ODc < ODA < ODc×2	Non adherent / Weakly adherent
ODc×2 < ODA < ODc×4	Moderately adherent
ODc×4 < ODA	Strongly adherent

NG=Negative control
ODc= The mean Optical density of negative control
ODA=The mean of three Optical densities of each *A. baumannii* isolate

DNA extraction

The genomic DNA of clinical isolates of *A. baumannii* was extracted using a commercial purification system (BIONEER/Korea) and the purity and concentration of the DNA were determined using a spectrophotometric instrument (NanoDrop) by measuring the optical density (O.D). The extracted DNA was then stored at -20°C for future use.

PCR reaction mixture

To prepare the polymerase chain reaction (PCR) mix for each primer (16srRNA, *CsuE1*, *and OmpA*) (Table 1), a final volume of 25 µl per reaction was used. This mix contained 1 µl of forward and reverse primers, 5 µl of pre-mix (master mix), and 15 µl of deionized water. To this mixture, 3 µl of *Acinetobacter baumannii* DNA was added.

Table (1): The sequence of *Acinetobacter baumannii* primers.

Primer	The Sequence of Primer F(5´- 3´), R (3´-5´)	PCR Product (b.p.)	Reference
16srRNA	F: AGAGTTTGATCCTGGCTCAG	1494	Arockia *et al*., (13)
	R: TACCTTGTTACGACTT		
ERIC	F: ATGTAGCTCCTGGGGATTCAC	variable	Gautam *et al.*, (14)
	R: AAGTAAGTGACTGGGGTGAGCG		
OmpA	F: GTTAAAGGCGACGTAGACG	578	Omid *et al.*, (6)
	R: CCAGTGTTATCTGTGTGACC		
CsuE1	F: CCAGACTGCCGAATTTTAGC	187	Al-Hadethi and Turki, (15)
	R: TATTTCCCGTTTGCGACTTC		

PCR Program

PCR tubes were transferred to the thermal cycle machine. The amplification program for each primer was started, and the amplification products were analyzed on a 2% agarose gel with the presence of a DNA ladder marker of 1500 bp.

Table (2): The program PCR amplification of *16srRNA, CsuE1* and *OmpA* gene.

Steps	Temperature(^{0} C)	Time	Cycle Number
Initial Denaturation	95 ^{0} C	5 min.	1
Denaturation	95 ^{0} C	30 sec.	35
Annealing of *16srRNA*	51 ^{0} C	30 sec.	
Annealing of *CsuE1*	56 ^{0} C		
Annealing of *OmpA*	56 ^{0} C		
Extension	72 ^{0} C	1 min.	
Final Extension	72 ^{0} C	5 min.	1

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and DNA amplification

Genotyping relatedness for clinical isolates of *Acinetobacter baumannii* was performed by ERIC-PCR using a pair of forward and reverse primers were employed in conventional PCR to achieve DNA amplification via ERIC-PCR., primes of (F) 5´-

ATGTAGCTCCTGGGGATTCAC - 3′ and (R) 3′ AAGTAAGTGACTGGGGTGAGCG-5′ (14). Were used, and a total volume of 25 l was used for the operation. The reaction component is indicated in (Table 3).

Table (3): The program that used in the thermos-cycler ERIC-PCR.

Steps	Temperature(^{0} C)	Time	Cycle Number
Initial Denaturation	95 ^{0} C	3 min.	1
Denaturation	95 ^{0} C	2 min.	5
Annealing	40 ^{0} C	2 min.	
Extension	72 ^{0} C	2 min.	
Denaturation	95 ^{0} C	1 min.	35
Annealing	52 ^{0} C	1 min.	
Extension	72 ^{0} C	1 min.	
Final Extension	72 ^{0} C	7 min.	1

Result and discussion

Collection of samples

The results revealed that only (50) isolates were given identical morphological characteristics and biochemical tests that belong to *A. baumannii* isolates. while the other (36) isolates seems to be related to different pathogenic genera, most of them were *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and the rest of them were belong different pathogenic genera, and the rest (64) 42% were negative samples (Table 4).

Table (4): Negative and positive growth according to type of sample

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

In this study, out of 50 positive cultures for *Acinetobacter baumannii*, 28 (56%) isolates were collected from burns, this result was agreed with the work of (16) 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.* (17), who shows a near percentage (17.3%) and (16%) for wound and sputum respectively. On the other hand, urine culture was 3 isolates only (6%) and this percentage was near to the percentage of the work of (18), which was (4%) for urine culture for *A. baumannii*. *Acinetobacter* infections were found as risk factors for mortality in (Figure 1) (19).

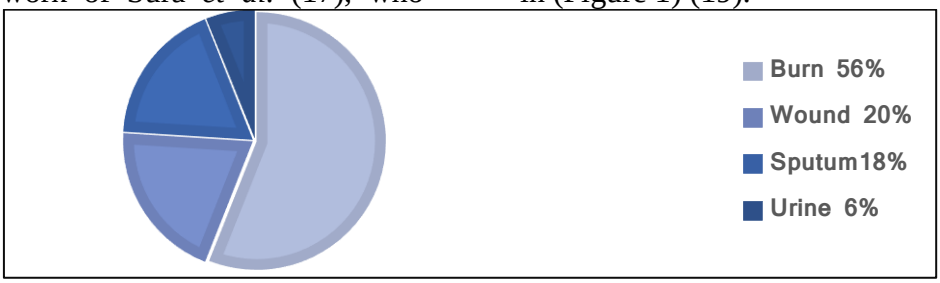

Figure (1): Distribution of *A. baumannii* isolates according to the type of sample

Identification of bacteria isolates

Each bacterial isolate was identified using a variety of morphological, microscopic, and biochemical tests, including culture on a specific medium called CHROMagar., morphological and microscopical characteristics. Then the characteristics of isolates were identified by some biochemical test and the identification was confirmed by using Vitek-2 system with accuracy 99%.

Gram stain can be used to identify probable isolated colonies of *Acinetobacter baumannii* based on their distinctive morphological characteristics, such as form, color, size, and organization. When examined under a light compound microscope, *A. baumannii's* Gram staining was visible, and bacterial isolates revealed themselves to be gram-negative bacteria, little coccobacilli that were occasionally organized in pairs and solitary coccobacilli. These findings were accepted Al-Ahmer *et al.* (20) microorganisms isolated from the environment were coccobacilli, gram-negative bacteria that sporadically formed diplococci.

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

CHROM agar *Acinetobacter* was designed as a highly selective medium, allowing the growth of *Acinetobacter* in conspicuously red colonies, after overnight incubation Moran-Gilad *et al.* (21). The *A. baumannii* isolates on the CHROM agar, appeared as bright red colonies after 24 hr. and incubation at 37°C.On the other hand, MacConkey agar, colonies of *A. baumannii* appeared as a non-lactose fermenter, lots of single circular colonies, smooth and transparent colonies. This result was agreed with the work of Raghda *et al*. (16) who mentioned that *A. baumannii* appear as small circular, Regular, smooth, mucoid and pale colonies and non-lactose fermenter on MacConkey agar. While on blood agar, *A. baumannii* colonies appear round gray in color, creamy colony and non-hemolyticThis result was similar with the work of Peymani *et al.* (22) who mentioned that *A. baumannii* colonies appear gray in color, smooth, about 1-2 mm in dimeter, creamy colony, circular and non- hemolytic due to absence of hemolysis enzyme.

The suspected isolates of *Acinetobacter baumannii* were then subjected to number of biochemical tests. All 50 isolates were found to be catalase and Simmon citrate test were positive for *A. baumannii* isolates positive, while Oxidase and indole test give negative result for *A. baumannii* isolates.

Vitek-2 System

In this identification, newly activated bacteria were cultured on MacConkey ager and incubated at 37°C for 14 hr. after incubation period a single colony of bacteria was transferred by using a plastic loop to 3 ml normal saline. Turbidity was measured by using McFarland spectrophotometer provided as parts of the device after the turbidity of the bacteria was standardize to 0.5 McFarland. The kit was added to every single tube and placed in the device. The results were obtained after 18 hr. of incubation in the device (23).

Biofilm production profile

According to the results, biofilm development was seen in all 50 (or 100%) of the isolates out of a total of (50). 40 (80.0%) of the tested isolates showed signs of strong biofilm, while 10 (20.0%) of them were able to produce moderate biofilms with an OD

of 630 nm and mean ELISA reader values that ranged from (0.1 to 0.2). On the other hand, there were (0.0%) no isolates that were identified as having poor biofilm formation, and no isolates that weren't producers were discovered.

Table (8): Biofilm-forming ability of *A. Baumannii* using micro-titer plate.

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

Another study showed that the result of biofilm formation in micro titer method were 4 isolate (28.5%) of *Acinetobacter baumannii* created moderately biofilm, 5 isolate (35.7%) were strong biofilm producer (24). Another study revealed that *A. baumannii* isolated from different clinical sources (wounds, burns, urine, sputum, blood and throat) were able to produce strong biofilm (25). The purpose of this study is to determine whether *Acinetobacter baumannii* can form biofilms, and it was (80.0%) Another study found that sensitive isolates could make strong biofilms for 24 hours, but afterwards their ability to form biofilms diminished and they could only form weak biofilms. Resistant strains, however, could form moderate to strong biofilms (26). According to National Institutes of Health and the Center for Disease and Prevention statistics, biofilms are linked to 65-80% of bacterial illnesses in people (27).

Molecular identification results of *A. baumannii*

Extraction of genomic DNA

All thirty *Acinetobacter baumannii* isolates were successfully genomic DNA isolated using a commercial genomic DNA purification kit (Bioneer Company/Korean) in accordance with the manufacturer's instructions. The results of the extraction were successful, and the Nanodrop spectrophotometer at 260/280 nm was used directly to quantify the DNA (concentration and purity). All of the isolates' extracted DNA concentrations ranged from (58 to 141) ng/l, and their purities were rated between (1.8 and 1.9). Extracted chromosomal DNA was verified and examined by gel electrophoresis in (0.7%) agarose for 60 min at 75 volts, and the DNA shows as compact bands under ultraviolet light. As shown in (Figure 2).

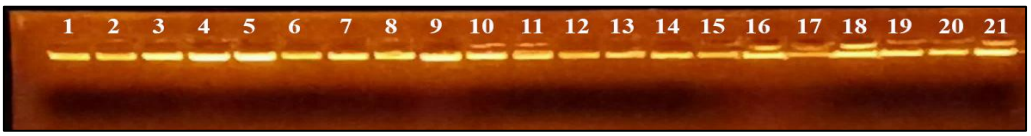

Figure (2): Gel Electrophoresis of Genomic DNA extracted from *A. baumannii* isolates on (0.7%) agarose, 75V, for 60 min stained with ethidium bromide

Molecular Detection of *A. Baumannii* by *16srRNA* Gene

By comparing the PCR products' molecular weight to a 1500 bp DNA ladder and analyzing the bands on gel electrophoresis, the results of the assay were validated. The *16srRNA* gene was shown to be present in all 30 (100%) DNA isolates of *Acinetobacter baumannii*. The figure 3 was shown all

clinical isolates identified as *A. baumannii*.

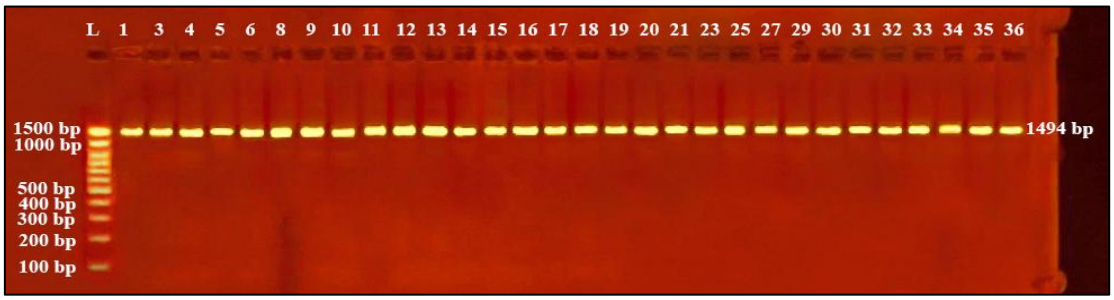

Figure (3): Gel Electrophoresis of 16srRNA gene product on (2 %) agarose gel, 75V, for 60 min. in presence of DNA Ladder (1500 bp)

Chaperone–Subunit E (*CsuE1*) gene

The results indicated that all examined *Acinetobacter baumannii* isolates have *CsuE1* gene (100%). The detection of Chaperone–Subunit E was carried out by using specific primers *CsuE1*-F and *CsuE1*-R, and the size of the amplified product was 187 bp length after electrophoreses on agarose gel (2%) in the presence of ladder marker 1500 bp as shown in figure (4).

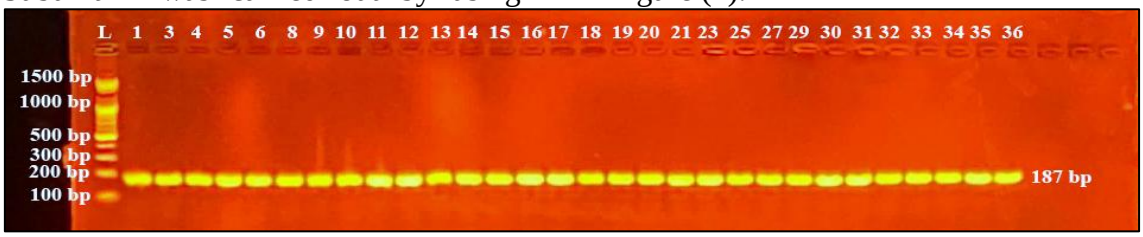

Figure (4): Gel Electrophoresis of *CsuE1* gene product (2%) agarose gel, 75V, for 60 min. in presence of DNA Ladder (1500 bp)

The current result was near to the work of Yang *et al.* (3) who reported that A higher biofilm formation was often generated by the multiple drug-resistant isolates. *CsuE1* and other biofilm-related genes were frequently present in the isolated isolates (91.6%). The current result agreed with the study (28) from Iraq, which recorded the same percentage (100*%)* of *Acinetobacter baumannii* isolates possesses a *CsuE1* gene. And was agreed was with the work of Azizi *et al.* (29) who consisted that *CsuE1* gene were detected in all *A. baumannii* isolates. Because hospitalized patients are more susceptible to medical devices, are exposed to more antibiotics, and are harboring colonizers of bacteria that form biofilms in the surrounding environment, biofilms are a problem in hospitals because they increase the risk of drug resistance in those patients and lengthen the duration of treatment (30).

Outer Membrane Protein (*OmpA*) gene

Outer Membrane Protein was detected in *Acinetobacter baumannii* isolates isolated from various clinical samples. The results exhibited that 30 isolates out of 30 isolates (100%) of *A. baumannii* isolates have the *OmpA* gene. The detection was achieved by the amplification of *OmpA* gene using The *OmpA* -F and *OmpA* -R primers. The size of the amplified fragment was 578 bp length after electrophoreses on agarose gel (2%) in the presence of ladder marker 1500 bp as shown in figure (5).

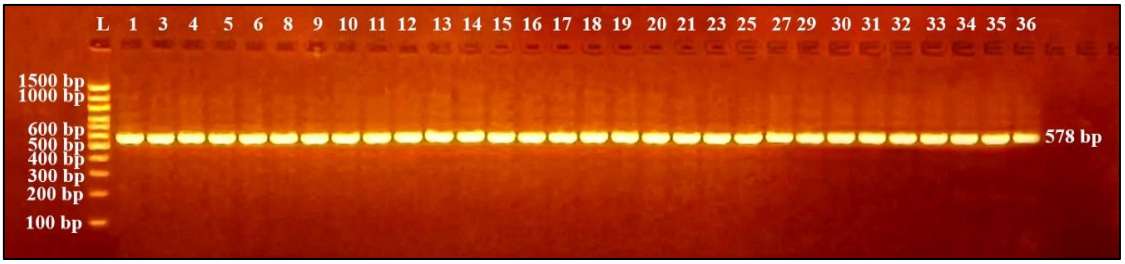

Figure (5): Gel Electrophoresis of *OmpA* gene product (2%) agarose gel, 75V, for 60 min. in presence of DNA Ladder (1500 bp)

The amplification of *OmpA* gene isolated from *Acinetobacter baumannii* was agreed with the work of Azizi *et al.* (29) who consisted that *OmpA* gene were detected in all *A. baumannii* isolates. and this result was similar with the work of Saba (28) who recorded that (100%) of *A. baumannii* isolates carry the *OmpA* gene. *OmpA* gene is the most prevalent surface protein among the outer membrane proteins found in *A. baumannii* (31). The main virulent factor of *A. baumannii, OmpA*, controls the host immune system as well as the adhesion, aggressiveness, and biofilm formation of the pathogen. An independent risk factor for the mortality rate of nosocomial pneumonia and bacteremia brought on by *A. baumannii* is the overproduction of *OmpA* (32).

Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and DNA amplification

Depending on the isolates, the ERIC-PCR banding patterns revealed 2 to 6 bands as shown in figure (6) and the amplicons varied in size from 200 bp to more than 1500 bp. The total similarity level of the ERIC-PCR fingerprinting grouped *A. baumannii* isolates from clinical samples was (74.1%).

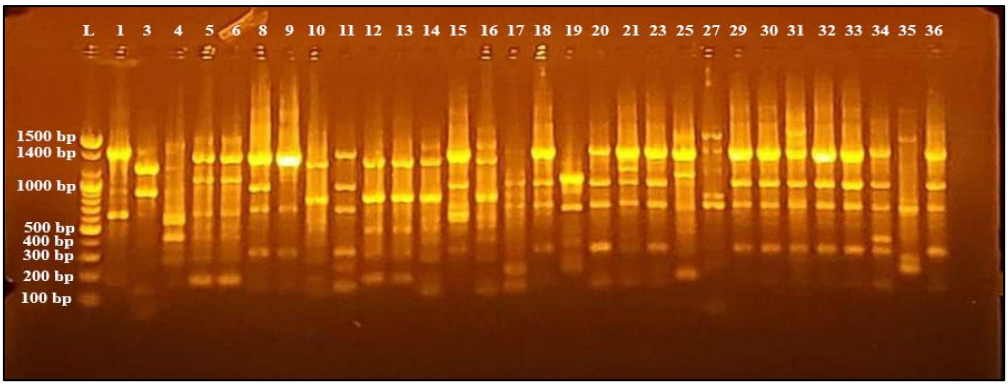

Figure (6): Gel Electrophoresis of ERIC-PCR product on (2%) agarose gel, 75V, for 60 min. in presence of DNA Ladder (1500 bp)

By using Gelquest software, the binding patterns from gel electrophoresis were used as the basic data to design a dendrogram. In this study UPGMA methodology was applied to provide us with phylogenetic tree as it depicted in figure (7). The ERIC-PCR fingerprinting grouped *Acinetobacter baumannii* isolates from clinical samples into many sub-groups according to band pattern for each isolates.

All isolates were grouped into two clades (clade 1-2) were observed at a similarity level about (74.1%) of all selected thirty isolates. This result was

near to the work of Aljindan *et al.* (33) how indicates that there was cross-transmission within hospitalized patients. According to our findings, ERIC-PCR is a valid approach for proving the clonal relatedness of *Acinetobacter baumannii* recovered from various specimens isolated from various inpatients.

Clade one (11) isolate which involve was observed at similarity level (89.25%). It was distributed from three sources (burn, sputum and wounds) and appeared to have the ability to produce strong biofilm on biotic and abiotic surface with optical density distributed between 375-682 nm. While Clade two (18) which involve was observed at similarity level (67.6%). The distributed from four sources (burn, sputum, urine and wounds) which appeared to have strong biofilm formation with optical density distributed between 301-704 nm.

The result of this study was similar to the work of Hammoudi *et al.* (34) used ERIC-PCR to group *Acinetobacter baumannii* strains according to their genetic similarity. One of the quickest molecular typing methods to distinguish *A. baumannii* from other strains of Gram-negative bacteria that because hospital acquired illnesses is ERIC-PCR fingerprinting Hammoudi *et al* (34) the genetic diversity of *A. baumannii* isolates was shown using ERIC-PCR. Several investigations employing various molecular typing techniques as well as ERIC-PCR have documented genetic diversity and variability among *A. baumannii* isolates. The ERICPCR method is generally recognized to be significantly more affordable and simple to use than other PCR-based typing techniques (35).

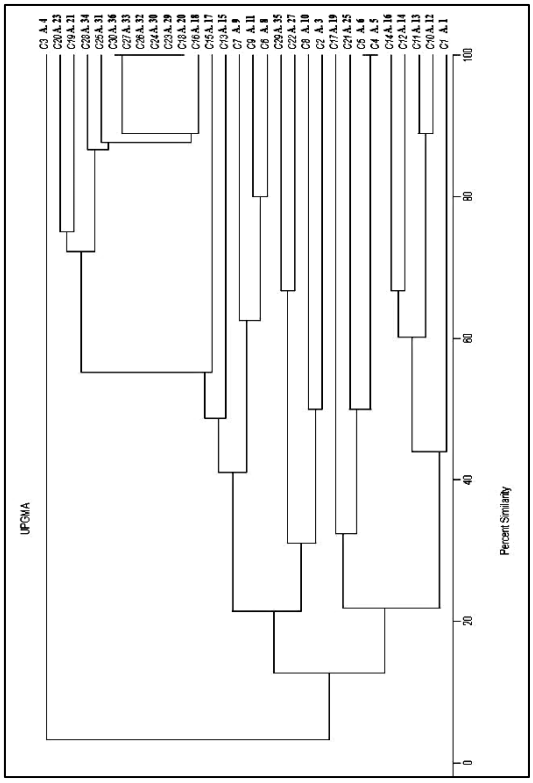

Figure (7): (UPGMA). dendrograms showing similarity level between 30 isolates derived from amplification pattern using (ERIC-PCR).

Table (10): ERIC-PCR report of A. baumannii dissimilarity

Imported data

CLUSTER ANALYSIS
Imported data
Analysis begun: Saturday, November 19, 2022 10:16:09 AM
Analysing 30 variables x 18 cases
Data will be transposed before analysis

UPGMA
Canberra

Node	Group 1	Group 2	Dissimil.	Objects in group
1	C4	C5	0.000	2
2	C18	C23	0.000	2
3	Node 2	C24	0.000	3
4	Node 3	C26	0.000	4
5	Node 4	C27	0.000	5
6	Node 5	C30	0.000	6
7	C10	C11	1.000	2
8	C16	Node 6	1.000	7
9	Node 8	C25	1.143	8
10	Node 9	C28	1.250	9
11	C2	C8	2.000	2
12	C6	C7	2.000	2
13	C12	C14	2.000	2
14	C19	C20	2.000	2
15	Node 10	Node 14	2.333	11
16	C1	Node 13	3.000	3
17	Node 11	C17	3.000	3
18	Node 12	C9	3.000	3
19	C22	C29	3.000	2
20	C15	Node 15	3.273	12
21	Node 16	Node 7	3.500	5
22	Node 17	C3	4.000	4
23	Node 1	C21	4.000	3
24	C13	Node 20	4.167	13
25	Node 18	Node 24	4.846	16
26	Node 21	Node 22	5.000	9
27	Node 26	Node 23	5.593	12
28	Node 27	Node 19	5.917	14
29	Node 28	Node 25	6.607	30

Conclusion

The present study showed that (100%) of the strong and MDR *A. baumannii* isolates have the virulence genes (*CsuE1 and OmpA* genes). The ERIC-PCR classified all selected isolates into two clades (clade 1-2) with different similarity level. ERIC-PCR shows that the similarity level of all selected isolates was observed at (74.1%). Our results demonstrate that 16srRNA, *CsuE1 and OmpA* gene PCR may be used to swiftly and easily identify *A. baumannii* isolates. Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) is good for epidemiological investigations and genetic diversity information.

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