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Molecular Typing of Extensively Drug Resistant *Acinetobacter baumannii* Clinical Isolates Using Arbitrarily Primed PCR (AP-PCR)

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Received: 3 September 2023 **Accepted: 8 October 2023**

DOI: https://dx.doi.org/10.24237/ASJ.02.03.815B

Abstract

Genotypic study of the strains is crucial for determining the genetic relationships between the strains and epidemiological investigations as well as for monitoring their geographic distribution. The goal of this study was to detect genetic similarities and differences between extensively drug resistant *Acinetobacter baumannii* isolates from patients in different hospitals in Diyala/Iraq by using Arbitrarily-PCR technique. The present investigation was conducted from October 2022 to March 2023. A total of 30 isolates of *Acinetobacter baumannii* were obtained from a sample size of 200 specimens. Isolates were identified using both traditional methods and the VITEK 2 compact system. The isolates was determined using the disc diffusion method. All *A. baumannii* isolates were resistance to (Piperacillin, Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone, Ampicillin–sulbactam, Piperacillin–sulbactam, Ticarcillin–clavulanic acid and Gentamicin) in percentage (100%) While resistance to Amikacin 96.7%. Imepenem and Meropenem was 76.7%, Levofloxacin was 66.7% and finally to Ciprofloxacin was 63.3%. Eleven(11) isolates (36.7%) were multi drug resistant, and nineteen (19) isolates (63.3%) were extensively drug resistant. The genetic variability of 19 extensively drug-resistant *A. baumannii* strains were investigated with the AP-PCR method, the genetic fingerprint has14 bands with molecular weights ranging from (300-4000) bp. AP-PCR typing showed a total of 19 XDR-*A. baumannii* isolates revealed 13 different clones with 90% similarity cutoff value include 6 groups of genotypes and 7 unique isolates. The present study

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concluded by demonstrating that the AP-PCR approach is a reliable, simple, quick, and cost-effective tool for examining the genetic diversity of *A. baumannii* isolates.

Keywords: *Acinetobacter baumannii*, Antibiotic Resistance, XDR, Arbitaly PCR, Genotyping.

التنميط الجزيئي للعزلات السريرية للراكدة البومانية شديدة المقاومة للمضادات الحيوية باستخدام تقنية تفاعل البلمرة المتسلسل من نوع (PCR (Arbitaly

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الخلاصة

ان الهدف من هذه الدراسة هو الكشف عن أوجه التشابه والاختلاف الجينية بين العزلات السريرية لبكتيريا *Acinetobacter baumannii* المقاومة للأدوية على نطاق واسع والمعزولة من مستشفيات مختلفة في محافظة ديالى / العراق، باستخدام تقنية تفاعل البلمرة المتسلسل من نوع (Arbitaly PCR).أجريت هذه الدراسة خلال الفترة ما بين أكتوبر 2022 ومارس 2023. من أصل 200 عينة سرسرية تم الحصول على 30 عزلة من بكتريا *Acinetobacter baumannii* . تم استخدام الطرق الميكروبيولوجية التقليدية ونظام التشخيص VITEK 2 لتشخيص العزلات. بوساطة طريقة انتشار الاقراص القياسية (كيربي باور) تم التحقق من مقاومة و حساسية عزلات بكتريا*A. baumannii* لمجموعة من المضادات الحيوية من مجاميع مختلفة. كانت جميع عزلات *A. baumannii* مقاومة لمضادات Ceftriaxone, (Piperacillin, Ceftazidime, Cefepime, Cefotaxime وبنسبة (Ampicillin–sulbactam, Piperacillin–sulbactam, Ticarcillin–clavulanic acid,Gentamicin 100% , بينما كانت مقاومتها لمضادAmikacin 96.7% لمضادي Meropenem و Imepenem بنسبة 76.7%, ولمضاد Levofloxacinبنسبة66.7% واخيرا كانت مقاومتها لمضاد Ciprofloxacin بنسبة 63.3% . احدى عشر (11) عزلة من اصل 30 وبنسبة (36.7%) كانت متعددة المقاومة للمضادات الحيوية (MDR) و تسعة عشر (19) عزلة من اصل 30 وبنسبة (63.3%) كانت شديدة المقاومة للمضادات الحيوية (XDR). اظهرت نتائج التحري عن التباين الوراثي لـ 19 عزلة من عزلات *A. baumannii*شديدة المقاومة للمضادات الحيوية بتقنية البصمة الوراثية AP-PCR ظهور 14 حزمة ذات أوزان جزيئية تتراوح بين (300-4000)bp. بينما أظهرالتنميط الجيني بتقنية AP-PCR ان من مجموع 19 XDR-*baumannii* ظهور 13 نسيلة مختلفة بنسبة تشابه 90٪ تتضمن 6 مجموعات من الأنماط الجينية و7 عزلات فريدة. استنتجت الدراسة الحالية بإثبات أن تقنية AP-PCR هي أداة موثوقة وبسيطة وسريعة وفعالة من حيث التكلفة لفحص التنوع الوراثي لعزلات *A. baumannii*.

الكلمات المفتاحية: الراكدة البومانية , مقاومة المضادات الحيوية, المقاومة الشديدة للمضادات، البصمة الوراثية , التنميط الجيني.

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Introduction

Acinetobacter baumannii is a significant pathogen in hospitals, causing a variety of infections with high rates of illness and death [1] .Clinical isolates of *A. baumannii* have exhibited a remarkable ability to resist antibiotic treatment due to both intrinsic resistance to antimicrobials and the acquisition of drug-resistant elements from other organism [2]. One of the most striking features of the *A. baumannii* genome is its high degree of genetic variability. This bacterium is known for its ability to rapidly evolve and adapt to new environments, and this is reflected in the diversity of its genome, in addition to numerous mobile genetic elements present in the genome, including plasmids, transposons, and integrons, which can facilitate the spread of antibiotic resistance genes [3]. The importance of molecular typing in understanding *A. baumannii* infections and the relationships between bacterial species cannot be overstated. Recent evidence has shown that molecular typing techniques are effective in identifying the primary cause of infections in hospital settings [4]. According to Nutman and Marchaim, 2019 [5], molecular typing methods can be divided into four main categories: amplification-based, fragment-based, sequence-based, and genomics-based methods. For typing Acinetobacter species, particular molecular markers are frequently utilized in genotyping procedures. These methods include RFLP (Restriction Fragment Length Polymorphism), AP-PCR (Arbitrarily PCR), RAPD (Randomly Amplified Polymorphic DNA Analysis), REP-PCR (Repetitive Extragenic Palindromic PCR), ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus PCR), and PFGE (Pulse-field Gel Electrophoresis) [6]. Symmetrical elements, which are repeating sequences that are typically found in the non-coding region of DNA, are present in *A. baumanni*. Nucleic acid amplification-based methods involve amplifying specific segments of nucleic acid by designing primers based on the microorganism's specific gene sequences of interest. This process can be used to accelerate the amplification reactions of the target microorganism, arbitrarily primed PCR (AP-PCR) considered one of the amplification based method [7]. In the differentiation of strains of pathogenic microorganisms, the arbitrary primed polymerase chain reaction (AP-PCR) technique has been useful in generating fingerprints and is considered an effective tool [8]. Genotypic and phenotypic resistance patterns are the main factors effecting progression of *A. baumannii* infections [9]. Accordingly, the purpose of this

work was to use the arbitrary-PCR approach to ascertain the antibiotic susceptibility and clonal connection of clinical isolates of *A. baumannii*.

Methods

Isolation and Identification of *Acinetobacter baumannii* Isolates

From October 2022 to March 2023, all clinical samples were collected from wounds, urine, sputum, and burn infections in Baquba teaching hospital and Al-Batoul hospital in Baquba/Diyala, Iraq. The isolates were identified by cultivating all samples on MacConkey agar, blood agar, and Acinetobacter Hi-Chrom agar. Additionally, biochemical assays were employed to aid in the identification process. Furthermore, the Vietik 2 compact system was used to diagnose these isolates [10].

Antibiotic sensitivity test

The antibiotic sensitivity test (AST) of all *Acinetobacter baumannii* isolates was performed following the method described by Benson [11]. Mueller-Hinton agar plates were prepared as per the instructions provided by the manufacturer. The inoculum colonies were prepared overnight, and a culture of the isolates was transferred to a 3ml tube of normal saline. The turbidity was then adjusted to a level of 0.5 McFarland. The isolate was interpreted as either sensetive, intermediate, or resistant to a particular antibiotics (Piperacillin, Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone, Imipenem, Meropenem, Ampicillin-sulbactam, Piperacillin-tazobactam, Ticarcillin-clavulanic acid, Gentamicin, Amikacin, Ciprofloxacin and Levofloxacin) by comparing the inhibition zone with the standards set by CLSI [12].

DNA Extraction

Genomic DNA was extracted from bacterial isolates using extraction kits ZR Fungal /Yeast/Bacterial DNA MiniPrep protocol according to manufactures company instructions (ZYMO) from USA. The concentration and purity of DNAsamples were measured by using NanoDrop (Nabi / korea).

DNA fingerprinting by Arbitrarily Primed PCR (AP-PCR)

The extensively drug resistant *Acinetobacter baumannii* isolates were fingerprinted by Arbitararly primed PCR (AP-PCR) using M13 universal primers as shown in Table (1) which produces a PCR product with variable bands (bp).

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Table 1: Primers used for Arbitrarily Primed PCR (AP-PCR)

PRIMER	OLIGO SEQUENCE (5-3)	PRODUCT SIZE BP	ANNEALING TEMP (°C)	REF.
M13 universal primers Arbitararily primed PCR (AP-PCR)	F: -GTAAAACGACGGCC AGTG- R: CAGGAAACAGCTATGACCATG	Variable	Annealing 1 (37), Annealing 2 (60)	13

The DNA of *Acinetobacter baumannii*, as well as the primers and PCR Master mix (Intron, Korea), which included Taq DNA Polymerase, deoxynucleotides dNTPs, Reaction buffer containing MgCl2, and Gel loading buffer, were melted at 4°C and subsequently subjected to vortexing and centrifugation to allow the substances to settle at the bottom of the tubes. The PCR mixture was set up in a final volume of 25 µl, and the DNA was amplified according to the instructions outlined in table (2).

Table 2: Mixture of the specific interaction for Gen diagnosis

PCR mix		**Volume**
Taq PCR Pre Mix		5µL
DNA template		1.5µL
Primer	Forward Primer	(1µL) 10 picomols /µL
	Reverse Primer	(1µL) 10 picomols /µL
Nuclease free water		16.5µL
Final volume		25 µL

Table 3: Thermal cycling condition used for M13 universal primers Arbitaly primed PCR (AP-PCR)

No.	Phase	Tm (°C)	Time	No. of cycles
1-	Initial Denaturation	94°C	5min.	1
2-	Denaturation -2	94°C	30sec.	8
3-	Annealing	37°C	1min.	
4-	Extension-1	72°C	2 min.	
5-	Denaturation -2	94°C	30sec.	35
6-	Annealing	60°C	1min.	
7-	Extension-1	72°C	2min.	
8-	Extension-2	72°C	7min	1

The amplified PCR products were detected by performing agarose gel electrophoresis on a 1.5 % agarose gel in TBE buffer (Promega, USA) supplemented with 5µl of Ethidium bromide (10mg/mL) (Promega, USA). The gel was subjected to electrophoresis at a voltage of 70 volts per centimeter squared for a duration of 90 minutes. The DNA bands were observed and captured through the utilization of ultraviolet (UV) light. The sizes of the products were compared to a MW100 and 1000 bp DNA ladder (Promega, USA) for the purpose of analysis.

Statistical analysis

Data are shown as percentages frequency,SPSS version 23 was used to perform the statistical analysis. Chi-square test was used to compared between percentages-Statistical significance was set at $P<0.05$. Dendogram analysis was done by using Numerical Taxonomy System (NTSYS-pc) program [14], to determine the genetic relationship between all extensively drug resistant *A.baumannii* isolates. The Dice coefficient and the unweighted pair group method with arithmetic averages (UMGMA) were employed to assess strain similarity and perform clustering, respectively.

Results and Discussion

Isolation of *A.baumannii*

The results of this study observed only Thirty (30) isolates of *A.baumannii* could be isolated from different clinical samples of these: 12 (40.0%) *A.baumannii* bacterial isolates were

identified from wound samples, 8 (26.7%) from burn samples, followed by 6 (20.0%) and 4 (13.3%) were identified from urine and sputum samples respectively (Figure1).

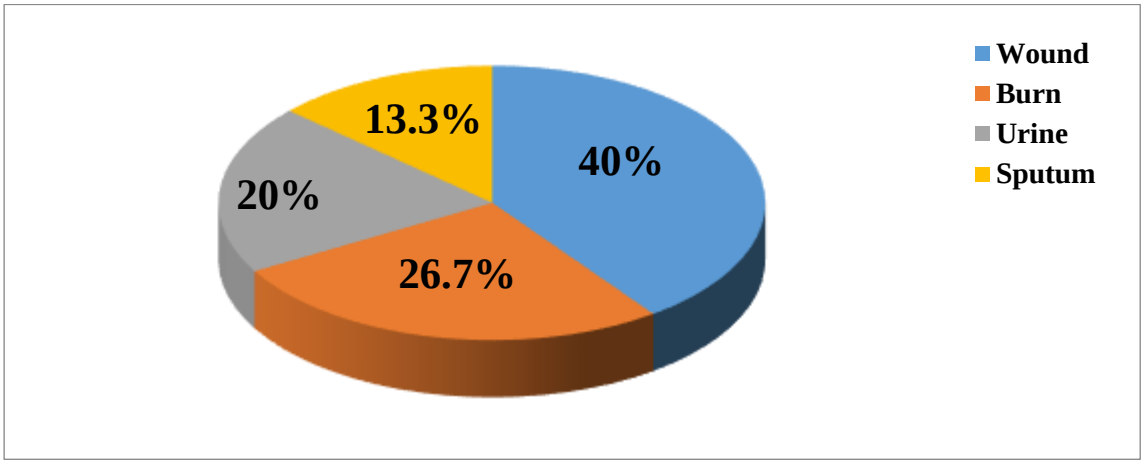

Figure 1: Distribution of *A. baumannii* isolates among different clinical samples

Antibiogram Patren of *Acinetobacter baumannii*

The result of antibiotic sensitivity test using the Kirby –Bauer method explained that all *A. baumannii* isolates were resistance to (Piperacillin, Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone, Ampicillin–sulbactam, Piperacillin–sulbactam, Ticarcillin–clavulanic acid, Gentamicin) in percentage (100%), while resistance to Amikacin 96.7%. Imepenem and Meropenem was 76.7%, to Levofloxacin 66.7% and finally to Ciprofloxacin 63.3% as shown in the figure (2). The very rapid spread of *A. baumannii* resistance to antibitics is propaply due to the wrong and irregular use of antibiotics. The percentage of Cephalosporins- resistance isolates was 100% of isolates was resistance to Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone which identified in this study. The resultsof the current study is in agreement with Iraqi study that condected in Al-Najaf City by Al-Ghazaly and Tuwaij [15] which reported that *A. baumannii* isolates were100% resistance to Cephalosporins. While the current results disagree with other local Iraq study in Erbil hospitals [16] that showed 86% resistance to Ceftazidime and 4% to Ceftriaxone and Cefepime.

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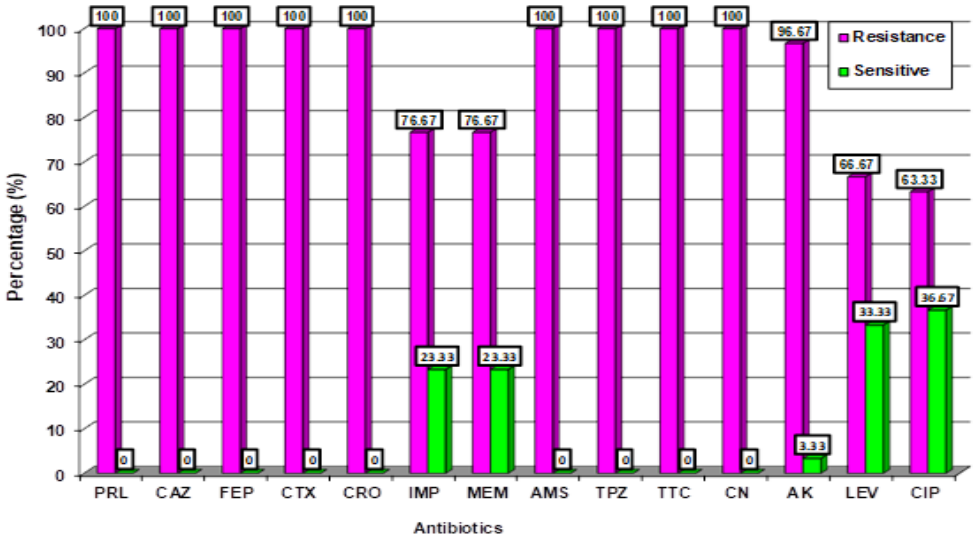

Figure 2: Antibiotic Susceptibility profile of *Acinetobacter baumannii* (n=30).

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

The present study showed that the isolates of *A. baumannii* displayed a high degree of antibiotic resistance, resistant to at least one antibiotic of three or more of the ten assessed antimicrobial classes in this study. According to this particular criterion, multi-resistant isolates were categorized into three groups: multi-drug resistant (MDR), maybe extensively drug-resistant (XDR), or possibly pan-drug resistant (PDR). Out of the total of 30 isolates, 11 (36.7%) were classified as MDR, while 19 (63.3%) were identified as XDR. It is important to acknowledge that the epidemiological importance of XDR bacteria arises from their resistance to a diverse range of antimicrobial medications and their distinctive tendency to acquire resistance against nearly all or the majority of antimicrobial treatments [17].

Arbitrarily Fingerprinting

The study employed the Arbitrarily-PCR genotyping technique to investigate the correlation among *A. baumannii* isolates of extensively drug-resistant obtained from burn victims, wounds,

sputum, and urine samples. The genetic profile of the extensively drug-resistant of *A. baumannii* isolates exhibits 14 distinct bands, with molecular weights ranging from 300 to 4000 base pairs (bp). These bands are observed at specific sizes: 300 bp, 400 bp, 500 bp, 600 bp, 800 bp, 900 bp, 1000 bp, 1100 bp, 1250 bp, 1500 bp, 1750 bp, 2000 bp, 3000 bp, and 4000 bp as show in Table 4 and figure 3.

Table (4): Results of *M13* gene for 19 XDR isolates

***M13* gene**	NO.	Percentage %
Positive	19	100
Negative	0	0.00
Total	19	100%
Chi-Square (χ^2)	---	16.533 **
P-value	---	0.0001
** (P≤0.01).		

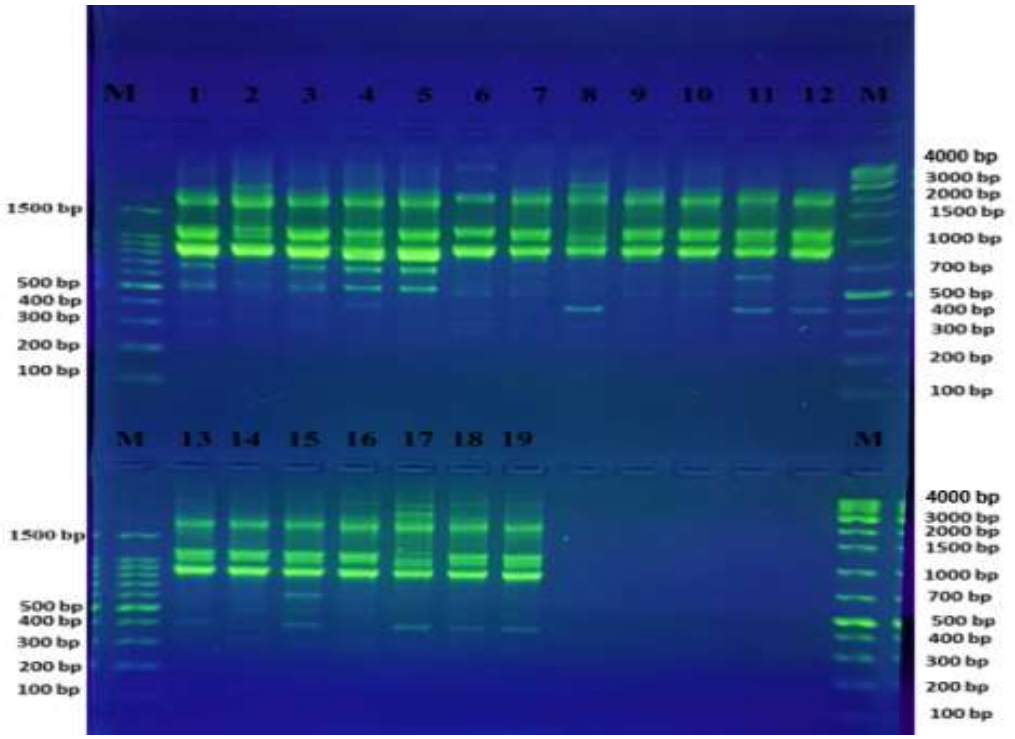

Figure 3: Patterns obtained by PCR with the M13 core sequence primer (1-19) *Acinetobacter baumannii* isolates, M: DNA ladder (100) and ladder (1000 plus).

The phylogenetic tree was generated using a computational algorithm and is presented in Figure 4. The study involved observing two primary groups, which were Group A and Group B. The AP-PCR typing method was employed to analyze a set of 19 isolates of XDR-*A. baumannii*. The results indicated the presence of 13 distinct clones, with a similarity cutoff value of 90%. These clones were further categorized into 6 groups of genotypes, while 7 isolates were found to be unique. Despite variations in the geographical distribution and separation sources for these isolates, a distinct clonality was evident, as depicted in Figure 4, suggesting the epidemiological relationship among these isolates. The collected strains were observed to be organized into two distinct clusters, denoted A and B. Within cluster A, two subgroups, A1 and A2, A1 consist of four genotypes (1,3,11,6) and A2 consist of four genotypes (2,16,9,10). 9 and 10 were 100% identical to each other .Cluster B was represented by two groups B1 and B2 , B1 consist of five genotypes (4,12,5,7,15), 4 and 12 were 100% identical to each other, while B2 consist of six genotypes (8,14,17,18,13,19).

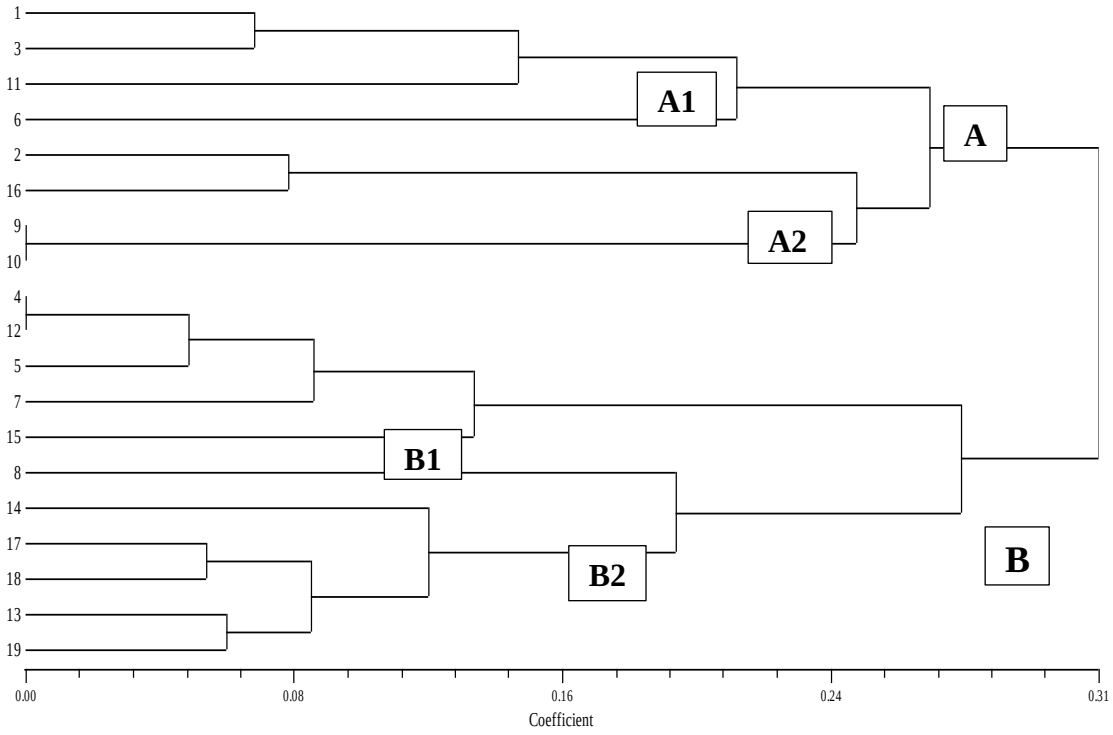

Figure 4: Arbitrarily Primed -PCR (AP-PCR) generated dendrogram showing genetic relatedness of *Acinetobacter baumannii* isolates to 19 extensively drug resistant

Arbitrarily Primed PCR is a PCR-based fingerprint techniques that is commonly favored due to its cost-effectiveness, simplicity, and rapidity in identifying and differentiating Acinetobacter species [7]. Therefore, it is imperative to prioritize infection control in healthcare facilities alongside the regular surveillance of antibiotic resistance trends across various geographical areas [18]. Özkul and Hazırolan (2021) conducted a study to examine the genetic links among 32 *A. baumannii* bloodstream isolates obtained from a hospital. The researchers employed AP-PCR analysis and identified six clusters of *A. baumannii*, consisting of six common kinds and five unique types [13]. In a study conducted by Gautam et al. (2022), the clonal diversity of 22 strains of *A. baumannii* originating from various clinics inside a hospital was examined. Through ERIC-PCR analysis, the researchers identified 15 distinct patterns, with 13 unique and 2 patterns shared among the strains [19]. To mitigate the dissemination *of A. baumannii* inside clinical environments, it is imperative to ascertain its sources and pathways of transmission [20]. In addition, AP-PCR analysis was conducted to ascertain the clonal relationship among the isolates. The observed variations in the heterogeneity of *A. baumannii*, as documented in multiple studies conducted globally, highlight the influence of environmental factors and hospital hygiene practices on this bacterium's distribution and genetic diversity. The clinical isolates of *A. baumannii* we examined had high drug resistance, specifically to a wide range of drugs. Furthermore, our analysis revealed significant genetic diversity and heterogeneity among these isolates, suggesting the involvement of multiple subgroups of the *A. baumannii* species in causing infections. Moreover, the existing findings suggest that AP-PCR genotyping could substantially impact routine epidemiological surveillance and identify the origin of *A. baumannii* infection within healthcare facilities.

Conclusions

This study found a high rate of extensively drug- resistant isolastes (XDR) for A. *baumannii* isolate. Finally, the current study demonstrated that the AP-PCR approach is a reproducible, simple, rapid, and cost-effective tool for examining the genetic diversity of *A. baumannii*

isolates. The results are important because the samples were collected from various hospitals and were closely related to one another.

Ethical Clearance: The experimental protocols conducted in this study received approval from the Department of Biology, College of Sciences, University of Diyala, in Diyala, Iraq. All experiments were conducted in adherence to the authorized standards. The scientific research conducted in Iraq is subject to ethical approval by both the Ministry of Health (MOH) and the Ministry of Higher Education and Scientific Research (MOHSER), as overseen by the Research Ethical Committee.

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