JJBS

Volume 15, Number 4,December 2022
ISSN 1995-6673
Pages 689 – 696
https://doi.org/10.54319/jjbs/150418

Jordan Journal of Biological Sciences

ERIC-PCR Genotyping and Clonal Genetic linkage Between Carbapenem-Resistant *Acinetobacter baumannii* isolates

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Received: December 15, 2021; Revised: March 1, 2022; Accepted: March 26, 2022

Abstract

Acinetobacter baumannii is a nosocomial bacterium that has become a global issue due to high levels of resistance to various antibiotics, notably last-resort treatments like carbapenems. The goal of this study was to use Clonal lineage and enterobacterial repetitive intergenic concensus - polymerase chain reaction (ERIC-PCR) finger-printing techniques to determine the source of the epidemic and the routes of *A. baumannii* isolates transmission. Sputum, blood, urine, burn wounds, and fluids from patients hospitalized to several hospitals in Baghdad, Iraq yielded 75 *A. baumannii* isolates. Morphological testing and PCR with *blaOXA-51* gene primers were used to identify *A. baumannii* isolates. The phenotypic results for *A. baumannii* isolate discovery were verified (100%) by molecular analysis. Twenty-nine *A. baumannii* isolates were categorized as multidrug-resistant (MDR), 36 as XDR, and eight as pan-drug resistant (PDR), whereas two isolates were responsive to most antibiotics. The lineages of the isolates were determined, with 8 belonging to group one, 6 to group two, and none to group three. 45 carbapenem-resistant *Acinetobacter baumannii* (CRAB) strains were grouped into six groups using ERIC-PCR fingerprinting genotyping analysis (selected only the 45 isolates resistant to carbapenem). The other seven strains were single strains. A significant level of genetic similarity was found amongst *A. baumannii* isolates from hospitalized patients, implying cross-transmission. The most likely explanation of the rise in *A. baumannii* occurrences in Baghdad patients was the spread of six epidemic clones, highlighting the importance of the study. To avoid nosocomial *Acinetobacter baumannii* spread, enhanced infection control methods are required. The findings further suggest that ERIC-PCR is a quick and accurate method for determining the clonal similarities of *A. baumannii* isolates from a variety of clinical samples.

Keywords: Clonal relationship, *Acinetobacter baumannii*, Genetic typing, Enterobacterial repetitive intergenic consensus – polymerase chain reaction.

1. Introduction

Acinetobacter baumannii has become one of the most difficult pathogens to control and treat among healthcare-associated illness pathogens (Szczypta *et al*.,2021). *A. baumannii* can cause a variety of infections, including bloodstream infections, surgical site infections, pneumonia, and urinary tract infections (Godziszewska *et al*.,2016). It has been discovered that it spreads in the hospital environment, causing epidemic outbreaks among hospitalized patients, due to its ability to colonize the skin, medical devices, and respiratory systems of patients and health care staff (Antunes *et al*.,2014). It can also form biofilm on surfaces that are not alive (Peleg *et al.*,2008). The ability to build biofilms and the presence of drug resistance mechanisms appear to be the greatest approaches to increase hospital mortality (Krzyciak *et al*., 2017).

Many drugs are naturally resistant to *A.baumannii*, and resistance can also be acquired by chromosomal changes and horizontal gene transfer (Pormohammad *et al*.,2019). The emergence of exceptionally drug-resistant (XDR) and multidrug-resistant (MDR) *A. baumannii* has become a public-health issue in both developed and developing countries (Salih & Shafeek,2019). Globally, resistance to last-resort antibiotics such as colistin and carbapenems is on the rise (Theriault *et al*.,2019).

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) outbreaks are common in healthcare facilities, particularly in intensive care units (Ridha *et al*.,2019). These outbreaks can be enhanced by clonal transmission or the spread of genetically unrelated strains. Clonality can be examined using molecular typing methods in such contexts, and several techniques have been used to type organisms in various environments (Alnimr *et al*.,2019). In the event of an incident, PCR-based genotyping schemes such as PCR-based sequence group typing, in which *A. baumannii* has been divided into three distinct groups, designated Group 1–3, based on their csuE (part of the pilus assembly system needed for biofilm formation), ompA (outer-membrane protein A), and blaOXA-51-like genes, are quick, cheap, and simple to use instruments for typing *A. baumannii*. In the same group of isolates, all three loci contained the same combination of alleles, indicating that the outbreak strains studied belonged to three primary clonal lineages. Using sequence changes across alleles, multiplex PCRs has been designed to

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quickly detect isolates that belong to specific genotypes or sequence groupings (Turton *et al*.,2007).

Enterobacterial repetitive intergenic consensus (ERIC) sequences are the most common repeated elements used for genetic typing (Ali & Khudhair, 2019). Non-coding transcribed areas are represented by ERIC sequences, which contain a higher central conserved inverted repeat (Yoke-Kqueen *et al*.,2013). The ERIC amplicon's sequence number and location are utilized as genetic markers to type and differentiate gram-negative bacteria (Omid *et al*., 2018). The goal of this study is to identify the origins of the outbreak and the transmission routes of *A. baumannii* isolates, which will be evaluated using clonal lineage and ERIC-PCR as a genetic typing technique.

2. Materials and Methods

2.1. Sampling

A total of 75 isolates have been taken from various hospitals in Baghdad (Central Children's Hospital, Baghdad Teaching Hospital, Burn Hospital, Children Protection Teaching Hospital (Medical City), Al-Imameen Al-Kadhimayn City Hospital) during three months from March 2021 to June 2021.The isolates included: 75 specimens taken from wounds, urine, respiratory infections (sputum), burns, and CSF of patients setting in hospitals, particularly in intensive care units. All the isolates were characterized via VITEK 2 System, the bio-chemical reactions between the bacterial isolates that have been suspended in their solutions, and media in VITEK2 Identification Cards. The specimens were transported to the laboratory (via transport media) and cultured on *A. baumannii*-selective media (Chrom agar) and MacConkey agar, incubated at a temperature of 37ºC for 24hrs under aerobic conditions. Then the colony's color, shape, edges, and texture were examined (Atlas *et al.*,1997).

2.2. Antimicrobial sensitivity test

The susceptibility of antibiotics to the antimicrobial agents listed below has been determined by using the disk diffusion approach on Mueller-Hinton agar (Merck, Germany) in accordance with the clinical and lab standards institute (CLSI 2020) Guidelines: meropenem (10µg), imipenem (10µg), cefixime (5µg), ceftazidime (30µg), cefepime (30µg), amoxicillin/clavulanate (20/10µg), amoxicillin (25µg), piperacillin-tazobactam (100/10µg), piperacillin (100µg), amikacin (30µg), gentamicin (10µg), Trimethoprim/sulphamethoxazole(25µg), ciprofloxacin (5µg), colistin sulfate (10µg) and tetracycline (30µg) were utilized. A fresh 24h bacterial lawn of the isolates was utilized for the preparation of 0.50 McFarland (1.50×10^8 CFU/ml) bacterial concentration. The bacterial growth inhibition diameter has been assessed and compared with reference tables that have been provided by the CLSI (CLSI, 2020).

2.3. DNA Extraction

Bacterial DNA extract was extracted from the Nutrient agar after 24 hours of incubation at 37ºC according to the boiling method as Annotated in Abed & Ali (Abed and Ali,2020). In a brief, 5 colonies have been thoroughly suspended in 1mL DNase- and RNase-free water and boiled for 10min. After centrifugation, five microliters of the supernatant were used as template DNA. DNA preparations were stored at - 20 C until they were needed for PCR.

2.4. Genotyping detection

It was confirmed by amplifying the blaOXA-51 gene for *A. baumannii* confirmation, detection by PCR technique in comparison with positive controls strain (Zueter & Harun, 2018; Ridha *et al*., 2019).

2.5. PCR - Based Sequence Group Typing

Multiplex PCR is used for the identification of the groups (international clones) of 1-3 organisms. This has been achieved with the use of primers given in Table 1 that were utilized for amplifying *csuE*, *ompA*, and *blaOXA-51-like* genes, which were applied for designating the sequence groups as well as the corresponding main international clones I–III as stated by Turton *et al*.(Turton *et al*.,2007). PCR was performed under the following conditions: 3 mins at 94ºC, then 30 cycles of 45sec at 94ºC, 45sec at 57ºC, and 1min at 72 ºC, followed by a 5mins extension at 72 ºC. For a strain to be classified as a member of group 1 or 2, it needed to have all three fragment amplifications in the corresponding multiplex PCR and no amplification in the other multiplex PCRs. PCR amplification of only *ompA* segment in group 2 and *csuE* and *blaOXA-51-like* fragments in group 1 was used to characterize the isolates in group 3 (Turton *et al*.,2007). The PCR products have been separated on 1% agarose gel and seen under ultraviolet light after being stained with ethidium bromide.

Table 1: Primers that have been utilized in multiplex PCRs for identifying sequence type groups

Primers	Sequence (5″ to 3″)	Size of the Amplicon (bp)
Group1 ompAF306	GATGGCGTAAATCGTGGTA	
Group1 and 2ompAR660	CAACTTTAGCGATTTCTGG	355
Group1 csuEF	CTTTAGCAAACATGACCTACC	
Group1 csuER	TACACCCGGGTTAATCGT	702
Gp1OXA66F89	GCGCTTCAAAATCTGATGTA	
Gp1OXA66R647	GCGTATATTTTGTTTCCATTC	559
Primer	Sequence (5″ to 3″)	Amplicon size (bp)
Group2 ompAF378	GACCTTTCTTATCACAACGA	
Group1 and 2 ompAR660	CAACTTTAGCGATTTCTGG	343
Group2 csuEF	GGCGAACATGACCTATTT	
Group2 csuER	CTTCATGGCTCGTTGGTT	580
Gp2OXA69F169	CATCAAGGTCAAACTCAA	
Gp2OXA69R330	TAGCCTTTTTTCCCCATC	162

2.6. Genotyping of Acinetobacter baumannii by ERIC-PCR

To investigate the genetic diversity and the clonal relationship of *A. baumannii* isolates, specific ERIC-PCR primers were used to identify the number of genetic patterns of isolates. ERIC-1(5′TGTAAGCTCCTGGGGATTCAC3′) and ERIC-2 (5′AAGTAAGTGACTGGGGTGAGCG 3′) were employed for this phase, as indicated via Coudron *et al.* (Coudron *etal*.,2000). The amplification reaction volume has been 25µl, while the cycling conditions were: After 5 minutes of denaturation at 94 ° C, 35 cycles of 1 minute at 95 ° C, 1min at 52ºC, 5min at 72ºC, and a final extension of 10min at 72ºC have been completed. Furthermore, the amplified products were separated by electrophoresis and stained with ethidium bromide on a 1% agarose gel. UPGMA was used to cluster the ERIC patterns, and the coefficient of Dice similarity has been utilized to compare them (Sallman *et al*.,2018).

3. Results

Over three months, 75 isolates of *A. baumannii* were collected from various hospitals in Baghdad/Iraq and genotypically and phenotypically confirmed as *A. baumannii* (Table2).

Table2: Distributions of *A. baumannii* strain specimens (*n* = 75)

samples	Number of isolates
Sputum	20 (26%)
Blood	18 (24%)
Burn	13 (17%)
Wound s.	10 (13%)
Urine	7 (9%)
CSF	4 (5%)
Fluid	3 (4%)
Total	75 (98%)

The phenotypic test results exhibited that all the isolates showed a pinkish tint colony due to non-lactose fermentation when cultured on MacConkey, while light purple with a halo around the colonies on chrom agar.

For confirming phenotypic identification regarding the *A. baumannii* isolates, PCR testing of the blaOXA-51 gene revealed that 75 (100%) isolates had the target gene (Figure1).

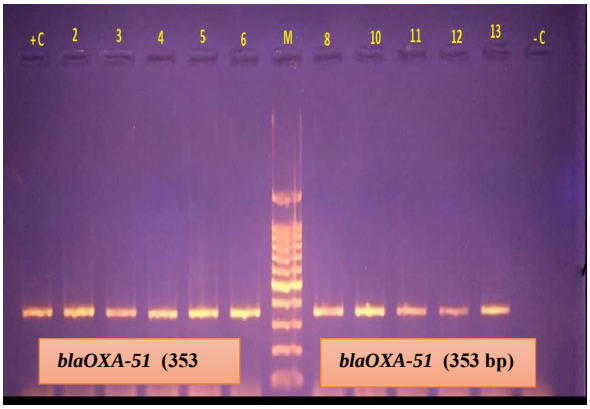

Figure 1: The blaOXA-51 gene product size (band 353 bp) was detected by using agarose gel electrophoresis (1% agarose, 7v/cm2) with Ethidium bromide staining. **Utilizing the** template DNA that was prepared with the use of the boiling method. The molecular size of the DNA ladder was 100 bp, located in the middle (M). DNA isolated from ***A. baumannii*** samples have shown positive PCR, with a positive control on the right and negative control on the left.

3.1. Antibiotic sensitivity test

The susceptibility profiles of 75 clinical isolates of *A. baumannii* to the antimicrobials studied were obtained in (Figure 2A). Among 75 *A.baumannii* with drug-resistant strains, most strains were resistant to common antimicrobial agents, although colistin was more effective. Interestingly, carbapenem appears to be a moderately effective antibiotic in this research. It is clear that *A. baumannii* isolates show a high level of resistance to piperacillin (98.66%) and amoxicillin (91%). The resistance pattern differed between samples from various sources, including 38.6 % (29/75) of *A. baumannii* isolates were classified as MDR, 48

%(36/75) as XDR, and 10.6 %(8/75) as pan-drug resistant. At the same time, two isolates 2.6% (2/75) were susceptible to fourteen of the sixteen antibiotics tested.

The dendrogram is based on the antimicrobial drug sensitivity pattern of seventy-five *A. baumannii* isolates. In this dendrogram, the isolate (63spu) was the origin of isolates responsible for resistance that utilized a 0. 4 cutoff to produce two clusters and then divided into different isolates with genetic variations. This indicated its ability to acquire different resistant genes as indicated in (Fig 2B).

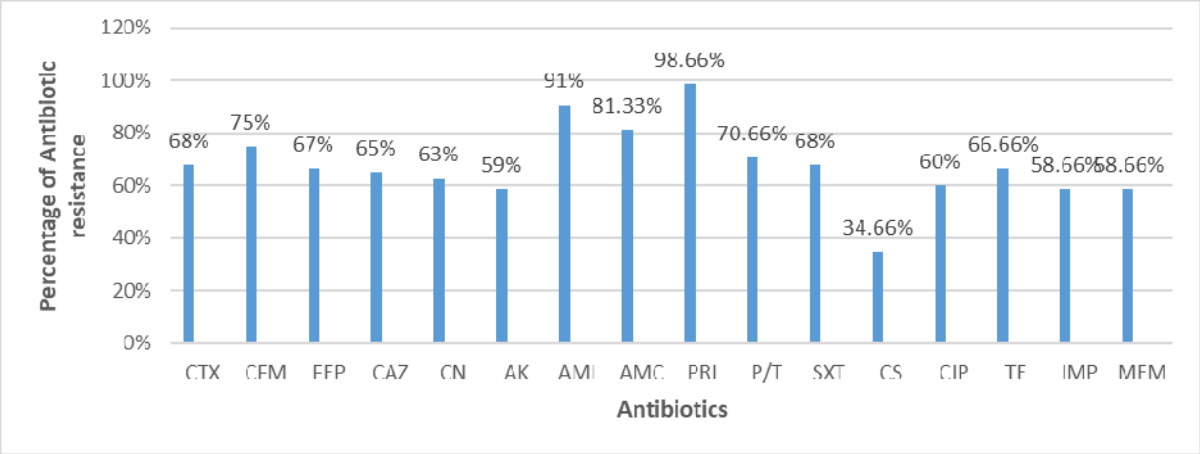

Figure 2A: **Antimicrobial resistance (%) of *A. baumannii* isolates.**

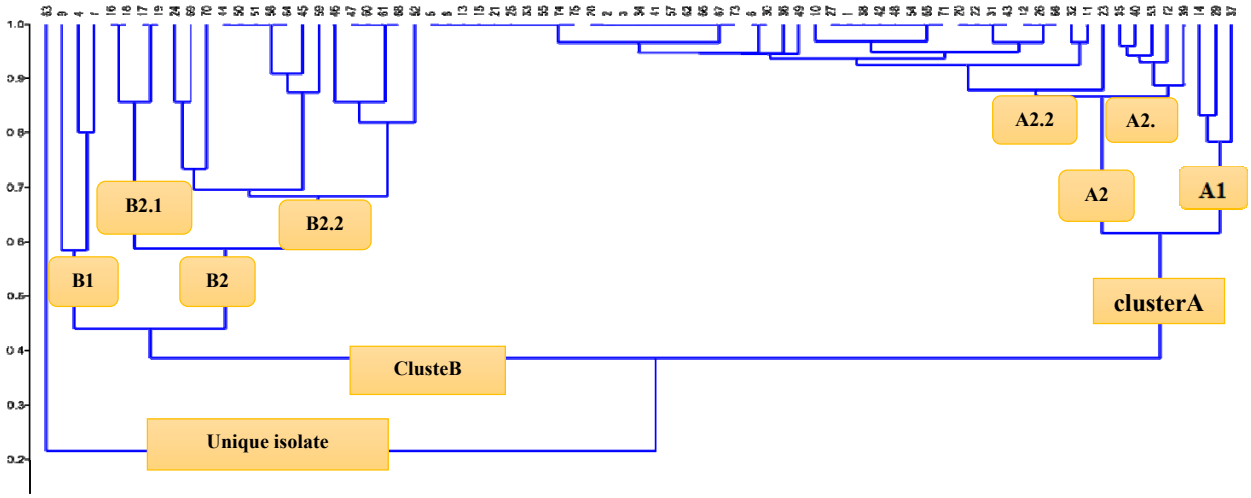

Figure 2B: Antibiogram typing analysis for 75 clinical *A. baumannii* isolates, by Dice coefficient values.

The accessions are obviously divided into two distinct clusters, A and B. Cluster A is divided into two subclusters, A1 and A2. The subcluster A1 consists of three isolates that were resistant five to eight antibiotics. The subcluster A2 is further divided into subgroups A2.1 and A2.2. The subgroup A2.1 consisted of five isolates which were resistant to three to sixteen antibiotics and subgroup A2.2 consisted of forty-three isolates which were resistant four to sixteen antibiotics. Cluster B also can be grouped into two subclusters B1 and B2. The subcluster B1 consists of three isolates which were resistant two to six isolates, while subcluster B2 is further divided into subgroups B2.1 and B2.2. The subgroup B2.1 consisted of four isolates which resist three to four isolates and the subgroup B2.2 consisted of sixteen isolates which resist two to fifteen antibiotics. Finally, all these clusters arise from a unique isolate (63 spu.) which was considered the origin of resistance.

3.2. PCR - based Sequence Group Typing

In group one of the clonal complex, all strains produced all three PCR fragments. However, there were none in group two PCR, while strains in group two gave the expected opposite results. Group 3 strains with the same ompA allele as Group2 produced the middle fragment (for ompA) in the Group2 PCR, yet the top two segments (for the other 2 loci) in Group1 PCR. Multiplex PCRs can thus be used to quickly classify outbreak strains into various sequence classes. Our findings revealed that eight isolates belonged to group one, six isolates to group two, and 39 isolates to group three. This method could not be used to type 22 isolates (Figure3) (Table 3).

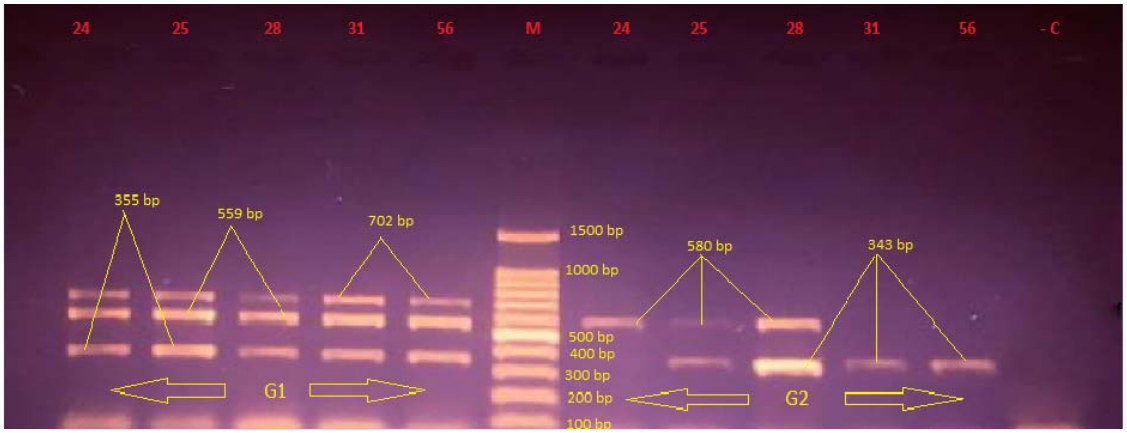

Figure3: Genetic detection of the global lineage through the multiplex PCR for 2 listed primers G1 *ompA (355 bp), csuE (702 bp) & blaOXA-51-like/ 66* (559 bp) and G2 *ompA (343 bp), csuE (580 bp) & blaOXA-51-like/66* (162 bp). Agarose Gel Electrophoresis (1% Agarose, 7V/Cm2) and Ethidium Bromide Staining to complete detection. In middle lane, molecular size DNA Ladder (100 bp DNA Ladder) and C indicate the negative control, isolates (24, 25, 28, 31 & 56) under G1 (ICI) due to harboring all G1 genes.

Table3: Determination of International Clone Types of *A. baumannii* isolates

Isolates number for each group with persentages	Isolates	Groups of international clone
22 (29.3%)	1CSF.,7 BL.,10 B.S, 14 spu.,15 BL.,16 spu.,18 W.S., 19 spu.,29 Uri.,30 spu.,34 spu.,35 BL. 36BL.,39 spu.,41 spu.,52BL.,59 B.S.,60 spu., 69Spu, 70 B.S, 71 BL., 76 B.S	G0
8 (10.6%)	12 W.S, 24 Ur., 25 BL., 28 Spu., 31 FL., 51 B.S, 56 Spu., 75 FL.	G1
6(8%)	3 Spu., 4 BL., 6 CSF, 21 BL., 45 B.S, 67 Ur.	G2
39(52%)	2 W.S, 5 B.S, 8 BL., 9 BL, 11 W.S, 13 CSF, 17 w.s, 20 BL., 22 BL., 23 Ur., 26 W.S, 27 W.S, 32 BL., 33 Spu., 37 Spu.,38 Spu. 42 Spu.,43 W.S,44 CSF, 46 Ur.,47 Ur.,48 B.S,49 FL.,50 B.S, 53Spu. 54 Spu.,55 Ur., 57 B.S,58 B.S, 61 W.S,62 B.S,63 Spu.,64 BL.,65 BL.,66 W.S, 68 W.S, 72 BL.,73 Spu.,74 BL.	G3

BL.: Blood, CSF: cerebrospinal fluid, Spu.: sputum, Uri.:urine. W.S.:wound swab, B.S: burn swab, FL.:fluid.

3.3. Amplification of DNA by ERIC – PCR

The ERIC-PCR fingerprinting technique has been utilized in order to classify *A. baumannii* strains obtained from different samples and at the same location and isolation period. It produced 17 DNA amplicon bands ranging from 100 to 1400 bp on average. Clonal diversity was discovered among 45 carbapenem-resistant *A. baumannii* isolates by detecting 13 ERIC patterns (ERIC - types), six of which were common type (A-F) that were divided into two clusters (Clusters 1–II) and seven unique types with a 97% similarity level. ERIC-type E was identified as the dominant type, with 11 isolates belonging to it (24.4%). In addition, genotype C had 5 isolates, followed by genotypes B and F (four isolates), and A and D (three isolates). Other isolates have been distributed in other patterns, revealing seven single types (between them, two isolates, 35 Flu. and 75 Flu., were considered the origin of types), and eight isolates were non-typed by ERIC PCR. Our results classified 30 (66.6%) isolates into six main genotypes (Figure4).

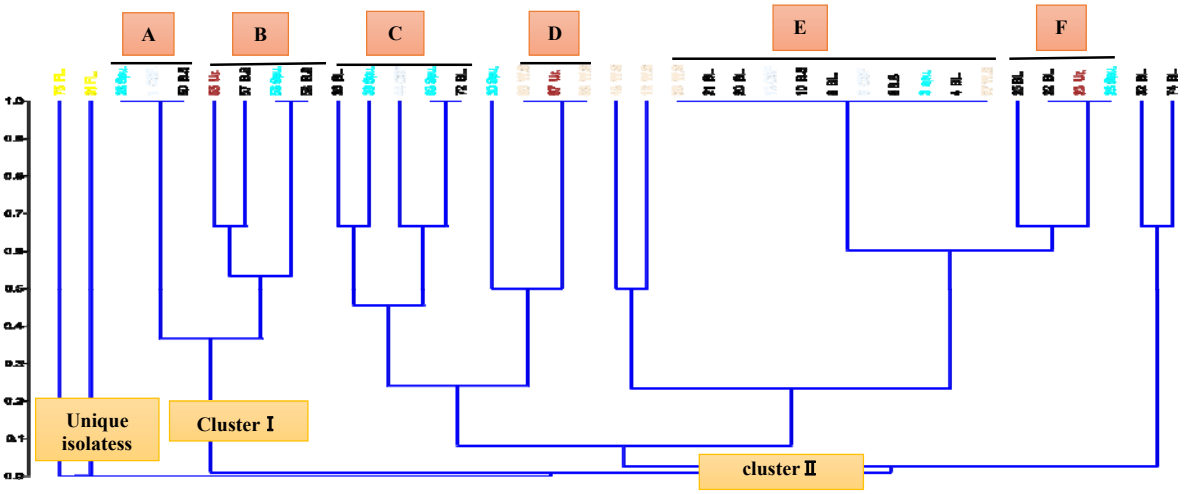

Figure 4: ERIC dendrogram utilizing the Bionumerics Fingerprint Analyst Software (Applied Maths) and unweighted pair groups, Dice technique, and arithmetic averages at 97% similarity on 45 *Acinetobacter baumannii* isolates.

4. 4. Discussion

A.baumannii is a potential nosocomial pathogen that causes a wide range of illnesses, from small soft tissue infections to more serious infections like bacteremia and ventilator-related pneumonia (Moubareck & Halat,2020). In this study, 75 *Acinetobacter baumannii* isolates were found in sputum, blood, wounds, burns, urine, fluids, and cerebrospinal fluid from patients with nosocomial infections. Our findings reveal that most isolates obtained were from sputum (26%) followed by blood (24%). This indicated that respiratory tract infections, which are typically connected with endotracheal tubes or tracheostomies, peritonitis in patients undergoing peritoneal dialysis, and catheter-related blood circulation infections were the most common types of *A. baumannii* clinical isolates. Our findings matched those of prior investigations by Saed *et al.* (2015) and Smail & Ganjo (2020). Mechanical ventilation, endotracheal intubation, and intravascular catheterization have all been linked to respiratory infections in previous research (Raka *et al*.,2009).

The rapid rise of MDR-AB and XDR-AB strains has become a serious problem in the treatment of hospital-acquired infections in recent years (Pournajaf *et al*.,2018). *A. baumannii* isolates developed resistance to the most routinely prescribed antimicrobial drugs, including aminoglycosides, cephalosporins, and extended-spectrum penicillins, in combination with -lactam inhibitors, quinolones, and carbapenems, according to our laboratory findings (Figure 3-A). The isolates were then categorized into three categories based on their antibiotic resistance pattern: MDR 38.6 % (29/75), XDR 48 % (36/75), and PDR 10.6 % (8/75) based on these findings. The drug of choice for treating the infection was carbapenems. However, *A. baumannii* resistance to carbapenem in clinical isolates has recently been identified as a serious danger, meaning that if carbapenem is misused, early treatment discontinuation will likely result in a rapid increase in resistance and treatment failure (Gango *et al*.,2016). Due to bacteria's ability to acquire antibiotic resistance genes and survive for days in the hospital environment and on the hands of health-care workers (HCWs), endemic *A. baumannii* strains may be transferred and persist in hospitals (Shalaby *et al*.,2016). Colistin is one of the most extensively used alternative medicines for carbapenem-resistant *A. baumannii*, according to Behera and his group study (Behera *et al*.,2017). Colistin is still one of the most effective single antimicrobial agents against multidrug-resistant *Acinetobacter baumannii*, and it's frequently employed as a last resort (Hatami,2018). These findings are similar to those of Xie *et al*. (2018) and El-Kazzaz *et al*. (2020).

A. baumannii is able to acquire antibiotic resistance genes and survive for days both in the hospital environment and on the hands of healthcare workers (HCWs), which could lead to possible transmission and the persistence of endemic *A. baumannii* strains in hospitals (Shalaby *et al*.,2016)

Previous studies indicated that each antimicrobial agent (except colistin) had a resistance pattern of greater than 50% against *A. baumannii* in various clinical specimens. Colistin sensitivity was determined in all clinical isolates (100 percent). Antibiotic overuse in hospitalized patients, as well as delays in discharge, organ implants, and prolonged catheter use, are all contributing to the spread of resistant bacteria among vulnerable patients (Amini *et al*.,2012)

Different molecular approaches have revealed genotypic diversity to track the incidence and evolution of drug-resistant bacteria (Khuntayaporn *et al*.,2021). Within *A. baumannii*, two molecular typing approaches, global lineage and enterobacterial repetitive intergenic consensus - polymerase chain reaction - were applied in our study (ERIC - PCR).

According to PCR analysis, all isolates were employed multiplex PCR with their stated circumstances to determine which type of clones belong to its two primer groups (Turton *et al*.,2007). Three intrinsic genes, *ompA, csuE, and blaOXA-51-like*, were targeted for this purpose.

In this study, 75 strains were divided into four groups: G1, G2, G3, and G0, with all strains from the G1 clonal complex producing all three segments in G1 PCR but none in G2 PCR, and strains from the G2 generated clonal complex producing the expected opposite results. Only the *ompA* allele, as well as *csuE* and *bla OXA-51* - alleles found in Group 1 PCR - are shared by Group 3 strains (Turton *et al*., 2007). However, the results of other strains did not reveal any of those genes, therefore they were quickly labeled as G0, which had never been documented before. As a result, the findings of the study revealed a significant prevalence of ICIII strains, with 52 % (39/75) belonging to this group. While the prevalence of each of the ICI was lower, reaching 10.6 %(8/75) for ICII and 8 % (6/75) for ICII, respectively. Finally, IC0 achieved a score of 29% (22/75). These findings contrast with those of Hamidian and Nigro, who found a greater dispersion of two major clones I and II on a large scale (Hamidian & Nigro,2019). The findings were also in contrast to those of Khuntayaporn et al. in Thailand, where recording ICII is far more common than recording ICI (Khuntayaporn *et al*.,2021). This difference in study outcomes could be attributed to a variety of factors, including geographical diversity.

Forty five carbapenem-resistant *A. baumannii* isolates were typed using ERIC-PCR. Two clusters (6 common kinds A-F) of *A. baumannii* strains with high relatedness, of which one ERIC-type predominated, were recovered from the same period of location and isolation, indicating that epidemiological relatedness has an impact on A. *baumannii* strain clustering (Fig. -4). Furthermore, the observed ERIC patterns revealed a high genetic similarity of 97 percent, as well as a higher number of DNA finger-prints among *A. baumannii* strains. Cross-transmission occurred among hospitalized patients, as evidenced by this finding. The conclusions of this study agree with those of previous studies (Hammoudi *et al*.,2015; Ying *et al*.,2015; Aljindan *et al*.,2018). Using the ERIC-PCR, researchers were also able to group *A. baumannii* strains based on genetic relatedness.

ERIC - PCR, according to our findings, is a quick and reliable method for demonstrating clonal relatedness of *A. baumannii* recovered from a variety of samples isolated from a variety of inpatients. As a result, compared to quick typing, this PCR-based target repetitive element approach is more suited, and it is also less expensive than other DNA fingerprint approaches.

5. Conclusion

A high level of genetic similarity was found amongst *A. baumannii* isolates from hospitalized patients, indicating cross-transmission. The most often discovered clonal lineage in the current investigation was international clone III (52 %, 39/75 isolates). This clone's isolates were largely linked to the *ompA* gene, which explains why 37.33 % of them carried the *ompA* gene. Furthermore, ERIC-PCR was used to indicate that the spread of six epidemic clones was the most likely source of the increase in *A. baumannii* occurrences in Baghdad patients. The findings also suggest that ERIC-PCR may be used to determine clonal similarities between *A. baumannii* isolates from a variety of clinical samples in a quick and reliable manner.

6. Ethical approval

The authors are confident that the publication of this manuscript will not raise any ethical issues.

Acknowledgment

The authors appreciate the support of Mustansiriyah University, College of Science, Biology Department, Baghdad, Iraq. for their contribution to this research work.

Conflict of interests

The authors declare that there is no conflict of interests in this publication.

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