Original Article

Classification of Environmental and Clinical Acinetobacter species Based on Some Antibiotics and Heavy Metals Resistant Genes

Evaluate Cadmium Resistance and Genetic Diversity in Acinetobacter Isolates

Ali Khanjar Jaber, Saba Riad Kkudhaier and Ali Murtatha Hasan

ABSTRACT

Objective: To evaluate cadmium resistance and genetic diversity in Acinetobacter isolates from clinical and environmental sources within hospitals.

Study Design: Experimental study

Place and Duration of Study: This study was conducted at the multiple hospitals in Baghdad City, Iraqi from 1st November 2024 to 31st March 2025.

Methods: Sixty Acinetobacter species isolates were collected; 44 A. baumannii isolates (41 clinical, 3 environmental) and 16 Acinetobacter complex isolates (13 clinical, 3 environmental). The isolates were identified using the VITEK-2 system, and the diagnosis was confirmed using polymerase chain reaction for the blaOXA-51 gene. 22 representative isolates were selected for further analysis. Cadmium resistance tests were performed using the minimum inhibitory concentration method, and the czcB gene was detected using polymerase chain reaction. Genetic diversity was analyzed using enterobacterial repetitive intergenic consensus-polymerase chain reaction and biotyping tools.

Result: All 22 isolates showed resistance to low cadmium concentrations (0.25mg/L, 0.125mg/L, and 0.0625 mg/L). The czcB gene was detected in 7 isolates (4 from A. baumannii and 3 from A. complex). Enterobacterial repetitive intergenic consensus-polymerase chain reaction demonstrated significant genetic diversity among the isolates. Cluster diagrams, heatmaps, and association matrices highlighted the phylogenetic relationships and unique characteristics of some isolates.

Conclusion: Acinetobacter isolates showed significant resistance to cadmium, partly attributable to the presence of the czcB gene. Enterobacterial repetitive intergenic consensus-polymerase chain reaction has proven effective in elucidating genetic diversity and tracking potential sources of infection.

Key Words: Acinetobacter baumannii, Acinetobacter complex, Cadmium resistance, Genetic diversity

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INTRODUCTION

The genus Acinetobacter, particularly A. baumannii, has emerged as a major cause of healthcare-associated infections due to its remarkable ability to survive in hospital environment and acquire resistance to antibiotics and disinfectants.[1]

These bacteria have also gained attention for their potential to tolerate and resist toxic heavy metals, including cadmium (Cd), which poses a serious environmental and health hazard due to its persistence and toxicity.[2]

In addition to phenotypic resistance, the genetic diversity of bacterial isolates is critical for understanding their adaptability and environmental persistence. The Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR technique is a widely used genotyping method to assess genomic variability among bacterial strains, including Acinetobacter spp.[3]

Cadmium is an SAM has no function in a plant, animal or human when its accumulation in college stays out, causing high blood pressure and kidney disease, and the difficulty to remove output Lead and Cadmium for direct damage neurons, because it prevents the formation of steel Colin and activates the enzyme choline esterase inhibitor) reduce the embarrassment of cadmium in the soil of 3-5 mg/kg this limit does not cause increased accumulation of a toxic concentration of lead occurring cadmium 5-10 mg/kg to reduce production and lay the seriousness of this element in it (SAM) in low concentrations.[4] Cadmium, often used in

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batteries and pigments, is a heavy metal. Stanford Advanced Materials (SAM) supplies various cadmium products, including cadmium acetylacetonate, cadmium sulfide powder, cadmium selenide powder, and cadmium telluride powder. SAM also offers cadmium metal, rods, evaporation materials, and sputtering targets.[6]

Generally, many studies had proven that when heavy metals present under traces were toxic to humans. Firstly, they combine with proteins and did not cause any poisoning, However, when concentration increase above the threshold level, they become a real health problem. These toxic metals could interact with important cellular components by forming covalent and ionic bonding, furthermore, when it became at high levels, both essential and non-essential metals causes cell membrane damage, enzyme specificity alteration, cellular function disruption and DNA structure damage.[5-8]

The purpose of this study is to evaluate the cadmium resistance of clinical and clinical environment Acinetobacter spp. isolates, detect the presence of the czcB gene, and assess their genetic diversity using ERIC-PCR.

METHODS

This experimental study was conducted at multiple hospitals in Baghdad City, Iraqi from 1st November 2024 to 31st March 2025 vide letter No. BCSMU/1221/00048M dated 1st September 2024. A total of 204 clinical and clinician environment samples were collected, comprising 136 clinical and 68 clinical environmental samples. Acinetobacter spp. was isolated from 54 clinical samples and 6 clinical environment samples. The clinical specimens were obtained from various sources including: wound swabs, burn swabs, sputum, urine, and blood, while the clinical environment samples were collected from operating rooms and outpatient clinicsas.

Diagnosis and identification of Acinetobacter species: All the isolates were cultured on ordinary media (MacConkey agar and blood agar) and incubated for 24 hrs at 37ºC. Depending on microscopically examination for Gram stain culture's characteristics, and biochemical tests (oxidase, catalase, kligler iron agar test) utilized for initial diagnosis. Identification was confirmed using theVITEK-2 system, and PCR amplification ofblaOXA-51gene. Cadmium susceptibility testing was performed using serial dilution. Detection of czcB gene was conducted by PCR. ERIC -PCR was used to evaluate genomic diversity. The specific primers for the study were confirmed by BLAST were detected by PCR Technique (Table 1). The chi-square statistical analysis was applied with significance at $p \leq 0.05$ to measure the significance between variables.

RESULTS

Collection and isolation of Acinetobacter species were categorized in Table 2. Acinetobacter spp. was isolated from 54 clinical samples and 6 clinical environment samples. The clinical specimens were obtained from various sources including: wound swabs, burn swabs, sputum, urine, and blood, while the clinical environment samples were collected from operating rooms and outpatient clinicsas (Table 3).

Table No. 1: Primer sequence of bla-oxa-51, ERIC, and czcB genes[6]

Gene	Sequences (5'-3')	Product size/bp
bla oxa 51	Forward: TAATGCTTTGATCGGCCTTG	353
	Reverse: TGGATTGCACTTCATCTTGG	
ERIC	Forward: ATGTAAGCTCCTGGGGATTCA	Variable
	Reverse: AGTAAGTGACTGGGGTGAGCG	
czcB	Forward: GCTGTGGCTGGAGATGAGAA	400
	Reverse: TTTTGCTCGGCATCCAAACG	

The clinical and clinician environmental strain isolates (n=60) of the isolated bacterial strains were in agreement with those reported for typical strains of 44 A. baumannii (41 clinical isolates and 3 clinician environmental isolates), and 16 A. complex (13 clinical isolates and 3 clinician environmental isolates), respectively. Therefore, after classifying 60 Acinetobacter spp. isolates (54 clinical isolates and 6 clinical environment isolates), based on phenotype (Biochemical tests, the Vitek-2 system, and confirmed through molecular identification by using the blaOXA-51 gene), we started with sequencing the 16SrRNA gene to determine the genetic identity of the isolates. However, due to the significant similarity between members of the Acinetobacter calcoaceticus baumannii complex (ACB complex), this analysis was not sufficient for precise differentiation. Therefore, we performed an analysis to detect the bla-oxa-51-like gene, which is a characteristic gene that is naturally present in A.baumannii as previously mentioned, this gene is considered a distinctive diagnostic genetic marker for the A.baumannii bacteria (due to the bla-oxa-51-like genes carrying up on a bacterial chromosomal specially). Through this, the isolates demonstrated morphological and genetic concordance with isolates of both A.baumannii and A. complex. based on this phenotypic and genetic concordance of the isolates (and its importance in providing high

accuracy in bacterial identification, which helps guide appropriate treatment and combat the spread of infection), we worked on reducing the number of isolates required to diagnose some antibiotic-resistant genes and heavy metals specific to this study. Therefore, we established a dendrogram Phylogenetic tree for the isolates of both Acinetobacter spp., a dendrogram typing for A.baumannii and a dendrogram typing for A. complex. Thus, the final isolates for the molecular study scientifically according to the theory of phenotypic and genotypic compatibility (Table 4, Fig. 1).

Table No. 2: Distribution of Acinetobacter spp. in clinical and clinical environment samples

Sample Type	Total samples	Negative culture (No growth)	Non-Positive for Acinetobacter spp.	Positive for Acinetobacter spp.
Clinical	136	9 (6.6%)	73 (53.7)	54 (39.7%)
Clinical Environment	68	46 (67.7%)	16 (23.5%)	6 (8.8%)
Total	204	55 (27%)	89 (43.6%)	60 (29.4%)

Table No. 3: Incidence of A. baumannii and A. complex isolates in various clinical and clinician environmental samples

Sources of Infection		A. Baumani (N, Rate of isolates %)	A. Complex (N, Rate of isolates%)
Clinical	Wound	12 (27%)	3 (19%)
	Burns	18 (41%)	9 (56%)
	Sputum	4 (9%)	-
	Urine	1 (2%)	-
	Blood	6 (14%)	1 (6%)
Clinician environment	Operating rooms	2 (5%)	2 (13%)
	Outpatient clinics	1 (2%)	1 (6%)
Total		44 (100%)	16 (100%)

Table No.4: Final Acinetobacter spp. isolates according to Dendrograms typing of 60 primary Acinetobacter spp. isolates

13 A. baumannii isolates	No.	9 A. complex isolates
A.b-Cli 1	1	A.c- Cli 43
A.b-Cli 2	2	A.c-Cli 56
A.b-Cli 17	3	A.c-Cli 42
A.b -Cli 25	4	A.c – En 46
A.b-Cli 27	5	A.c -Cli 53
A.b- En 29	6	A.c-Cli 49
A.b-Cli 30	9	A.c-Cli 57
A.b-Cli 32		A.c- En 48
A.b-Cli 36		A.c-Cli 55
A.b-Cli 40		
A.b-Cli 41		
A.b -En 47		
A.b-Cli 54		

Table No. 5: Cadmium resistance profile of A. baumannii and A. complex isolate

Tendency	S	D1	D2	D3	D4	D5	D6	D7	D8
A. baumannii (n= 13)									
Resistance of Cd^{2+} (Growth)	-	-	-	-	-	-	7 (53.8%)	13 (100%)	13 (100%)
Susceptible of Cd^{2+} (No growth)	13 (100%)	13 (100%)	13 (100%)	13 (100%)	13 (100%)	13 (100%)	6 (46.2%)	-	-
A. complex (n= 9)									
Resistance of Cd^{2+} (Growth)	-	-	-	-	-	-	7 (77.8%)	9 (100%)	9 (100%)
Susceptible of Cd^{2+} (No growth)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	2 (22.2%)	-	-

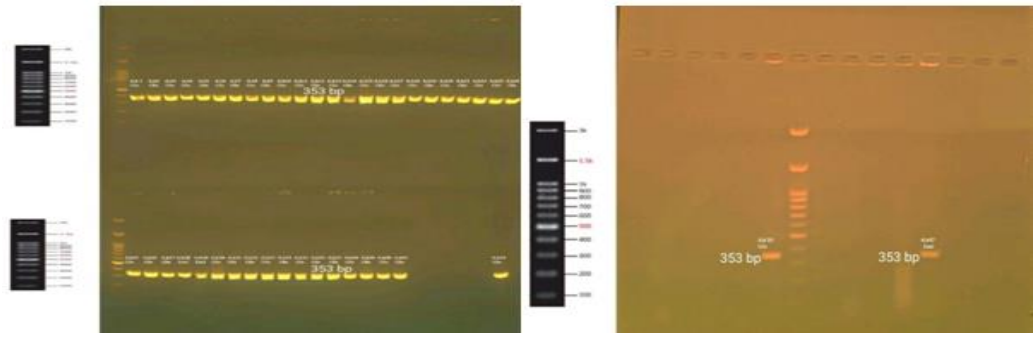

Figure No. 1: The amplification of the blaOXA-51 gene of A.baumannii samples was fractionated on 1.5% agarose gel (60 min at 7v/cm^2), (353 bp amplicon) M: 100bp ladder marker

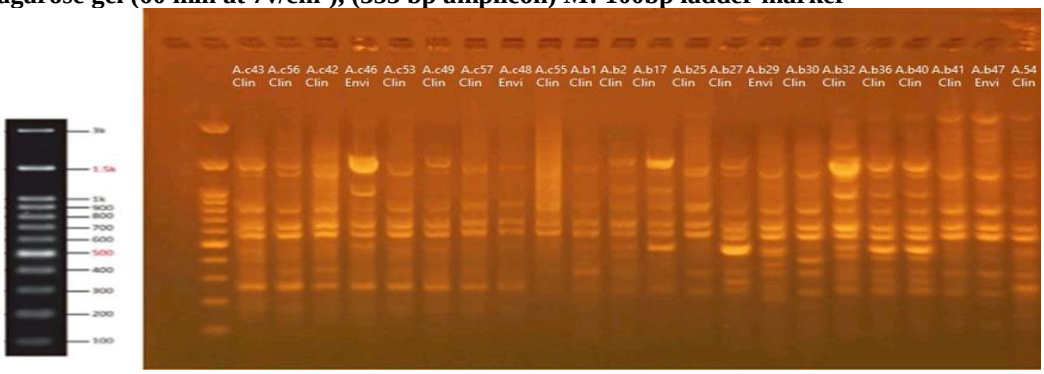

Figure No. 2: The amplification of the ERIC geneof Acinetobacter speciessamples was fractionated on a 1.5% agarose gel (60 min at 7v/ cm^2), (variable amplicons) lane: 100bp ladder marker

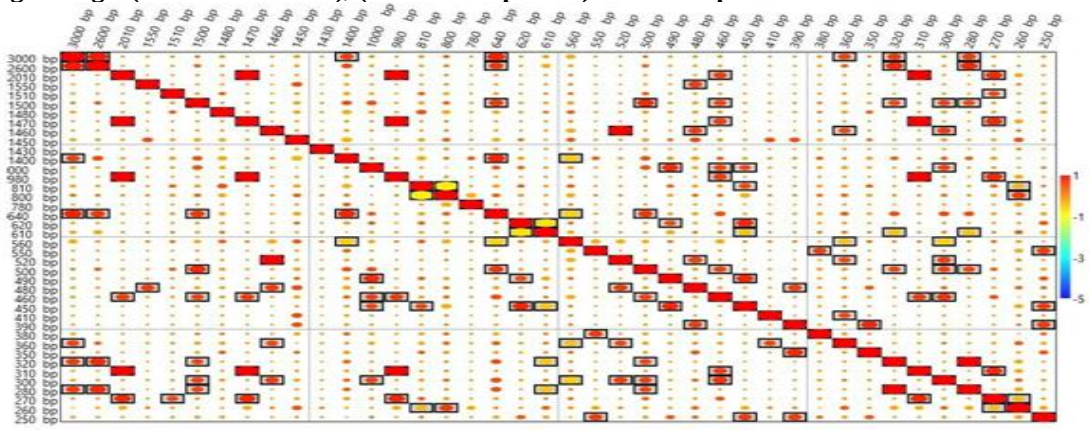

Figure No. 3: The Pearson matrix is a statistical method for determining the linear correlation coefficient between variables in ERIC gene

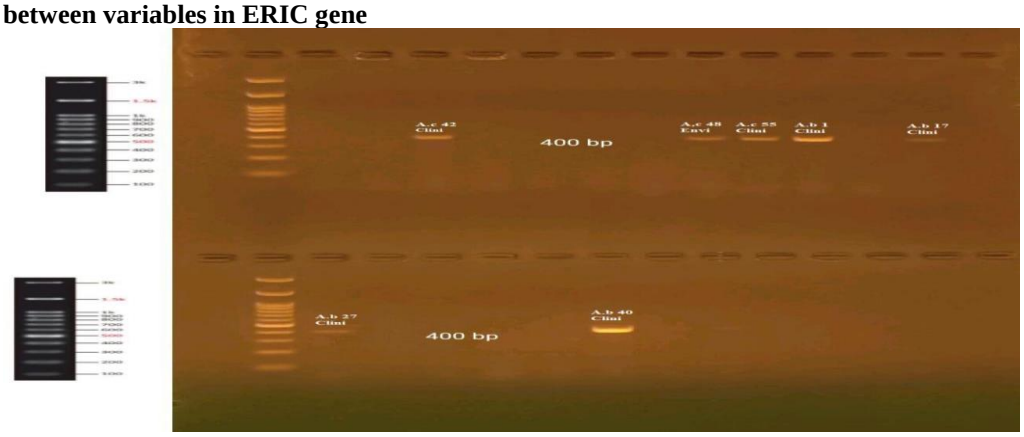

Figure No. 4: The amplification of CzcB geneof Acinetobacter species samples was fractionated on 1.5% agarose gel (60 min at 7v/ cm2), (400 bp amplicon) lane: 100bp ladder marker

Molecular Detection of Genetic Variation ERIC Gene: The Enterobacterial Repetitive Intergenic Consensus-PCR amplification of A. baumannii and A. complex produced unique polymorphic banding patterns, as seen in the agarose gel electrophoresis in Figure 2. Significant genetic variety among the strains was indicated by the numerous DNA bands of different sizes that were seen in the ERIC-PCR profiles of all 22 isolates. The band sizes varied between roughly 250 bp to 3 kb. Importantly, a variety of isolates showed strong bands at 590 bp, 610 bp, 800 bp, and 1400 bp, suggesting that these areas are frequently amplified between various strains. On the other hand, only a small number of isolates had particular bands, like those at 280 bp, 450 bp, and 1430 bp. Different isolates had different band numbers and extents, which could be due to strain-specific insertions or deletions or genomic rearrangements. To further analyse the dendrogram that displays the distribution of ERIC amplicons. This heat map representation illustrates the genetic fingerprinting of each isolate based on the observed bands. The X-axis represents different DNA fragment sizes, while the Y-axis lists the various isolates, which are represented by black squares indicating the presence of specific DNA fragments, while white spaces denote their absence. A strong correlation existed between the dendrograms and gel electrophoresis banding patterns.

Here we observe in the linear Pearson matrix, for example, the band for isolates of molecular size (3000bp) repeats or is associated with several bands, as the band for isolates of molecular size (3000bp) has repeated with, or is associated with, the band of molecular size (1400bp), and the band of molecular size (640bp), and the band of molecular size (360bp), and the band of molecular size (320bp), and the band of molecular size (280bp), but it is closely associated with the band of molecular size (640bp) (the size and position of the red circle inside the rectangle is very large, as it occupies most of the rectangle's size) [Fig. 3].

The presence of the heavy metal resistance czcB gene in 22 Acinetobacter spp. isolates as 13 A. baumannii isolates (11 clinical isolates and 2 clinical environment isolates), and 9 A. complex isolates (7 clinical isolates and 2 clinical environment isolates), was detected using PCR. The amplification of the czcB gene resulted in a distinct 400 bp band, confirming its presence in the positive isolates (Fig. 4).

The wells diffusion method, which involved serial dilutions (9-fold) of different cadmium concentration (Stock: 16 mg/l, D1: 8 mg/l, D2: 4 mg/l, D3: 2 mg/l, D4: 1 mg/l, D5: 0.5 mg/l, D6: 0.25 mg/l, D7: 0.125 mg/l, and D8: 0.0625 mg/l respectively), was used to evaluate the cadmium resistance. It was observed that all isolates resisted cadmium at the specified concentrations (D6, D7, and D8), including the isolates that appeared in the molecular (genetic) examination with the presence of the gene czcB. The results were identical in both examinations (genetic and phenotypic). The other isolates also showed resistance to cadmium in the phenotypic examination, but did not show genetic resistance to cadmium associated with this gene. We believe that these other isolates possess another gene within the czcABC system, such as the gene czcA or the gene czcC, or czcE. All 22 isolates did not grow at high concentrations (≥ 0.5 mg/l), but grew at the following concentrations 0.25 mg/l (D6): A. baumannii = 7/13 (53.8%), A. complex = 7/8 (77.8%). All isolates grew at 0.125 mg/l (D7) and 0.0625 mg/l (D8) = 100% growth. Statistical analysis indicates no significant difference in cadmium resistance between A. baumannii and A. complex at 0.25 mg/l ($P>0.05$), indicating similar levels of resistance at lower concentrations (Table 5).

DISCUSSION

The strong correlation between phenotypic and genotypic characteristics resulted in reducing the number of isolates from 60 to 22 representative isolates, helping to reduce costs and analysis time. The absence of significant statistical difference suggests that cadmium resistance may not be solely dependent on species type. ERIC-PCR revealed considereable genomic diversity, consistent with previous reports. czcBgene is positive in 4 of 13 A. baumannii isolates. 3 of 9 A. complex isolates.[9-12]

There are other isolates possess another gene within the czcABC system, such as the gene czcA or the gene czcC, or czcE. Evaluating cadmium resistance in Acinetobacter species, specifically A. baumannii and A. complex, provides important insights into how these bacteria respond to heavy metal stress. The relationship between DNA sequence and form is frequently referred to as the genotype–phenotype map (GPM), a term that is used in quantitative genetics about the effects of specific genomic regions on traits of interest. Likewise, in medicine, the GPM may refer to the association between mutations at specific loci and a disease or other condition. Given the complexity of form, researchers have sometimes sought to understand principles of the GPM by looking at specific processes such as RNA folding.[13-15]

CONCLUSION

Acinetobacter species showed resistance to cadmium in various and specific concentrations that represented possible adaptations in the environment by using some genetic factors that may affect metal tolerance. The Resistance to heavy metals in Acinetobacter species is mostly mediated by CzcB genes that encode to the CzcCBA efflux system. ERIC-PCR analysis provided insights into the genetic diversity among the isolates.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Ali Khanjar Jaber, Saba Riad Kkudhaier
Drafting or Revising Critically:	Ali Khanjar Jaber, Ali Murtatha Hasan
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

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