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Characterization of XDR-*Acinetobacter baumannii* clinical isolates: prominent role of *adeB* and *bla*OXA-51 in carbapenem resistance

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Authors:
AmirHasan Kavianpour[1]
Amin Talebi Bezmin Abadi[1*]

1. Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

**** Corresponding author:***
Amin Talebi Bezmin Abadi

ORCID: 0000-0001-5209-6436
Email: Amin.talebi@modares.ac.ir
Tel/ Fax: +982182884883

ABSTRACT

Introduction: Extensively drug-resistant (XDR) Acinetobacter baumannii is a global healthcare threat. This study characterized clinical XDR A. baumannii isolates from a Tehran hospital, focusing on carbapenem resistance mechanisms, virulence genes, and correlation with meropenem MICs.

Methods: Forty XDR *A. baumannii* isolates underwent phenotypic testing (mCIM, eCIM, DDST, meropenem MICs). PCR detected carbapenemase genes (blaOXA-51, blaNDM, blaVIM, blaIMP), ISAba1 upstream of blaOXA-51, and virulence genes (adeB, carO). qRT-PCR compared *adeB* and *carO* expression in high- vs. low-MIC isolates.

Results: All isolates were metallo-β-lactamase negative. blaOXA-51 was present in 95% (38/40), blaNDM in 20% (8/40); blaVIM, blaIMP, and ISAba1 were absent. adeB and carO occurred in 87.5% and 77.5%, respectively. All adeB-negative isolates belonged to the low-MIC group. Mean expression of *adeB* and *carO* was higher in high-MIC isolates, but differences were not significant ($p>0.05$).

Conclusion: Carbapenem resistance in these XDR isolates is primarily associated with intrinsic blaOXA-51 and acquired blaNDM. The high prevalence of adeB and carO suggests their contribution to resistance, but the lack of significant correlation with MICs indicates multifactorial, complex regulatory mechanisms beyond transcriptional activity.

Keywords: *Acinetobacter baumannii*, XDR, antibiotic resistance, gene expression, *bla*OXA-51, *adeB*, *carO*.

Introduction

The emergence and global spread of antibiotic-resistant bacteria pose one of the most critical public health challenges of the modern era, a crisis intensified by the widespread overuse of antibiotics in clinical and agricultural settings (1-3). In response, the World Health Organization (WHO) has repeatedly emphasized the urgent need for coordinated global action, identifying multidrug-resistant (MDR) infections as a top priority. Within this alarming landscape, *Acinetobacter baumannii* (*A. baumannii*) has emerged as a pathogen almost synonymous with antimicrobial resistance. As a nosocomial bacterium, *A. baumannii* presents a serious threat in hospital environments due to its ability to establish persistent infections, particularly among critically ill and immunocompromised patients (4). Over the past two decades, it has evolved from a relatively commensal into one of the most formidable hospital-associated pathogens worldwide (5-7). Its extraordinary capacity to survive on abiotic surfaces, persist under harsh conditions, and rapidly acquire resistance determinants has fueled its widespread dissemination. Consequently, carbapenem-resistant *A. baumannii* is now designated by the WHO as a critical priority pathogen requiring urgent development of new antibiotics (8). Numerous hospital outbreaks have highlighted the convergence of extensive antimicrobial resistance, high transmissibility, and key virulence factors, underscoring the multifaceted challenge posed by this pathogen (9). Of particular concern is the increasing prevalence of extensively drug-resistant (XDR) *A. baumannii*, defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. Infections caused by XDR strains are frequently resistant, leaving few therapeutic options and leading to prolonged hospitalizations, increased morbidity, and elevated mortality (10). Given the limited pipeline for novel antimicrobials, there is an urgent need to elucidate the specific molecular mechanisms underlying this phenotype (11). Carbapenem resistance in *A. baumannii* is predominantly mediated by carbapenem-hydrolyzing class D β-lactamases (CHDLs). Among these, *bla*OXA-51-like genes are intrinsic to the species and serve as a reliable molecular marker (12). Their overexpression, often driven by the insertion of the ISAba1 element upstream of the gene, is a major contributor to high-level resistance. Additionally, the acquisition of metallo-β-lactamases (MBLs) such as *NDM*, *VIM*, and *IMP* further compromises treatment efficacy. Beyond enzymatic degradation, important non-enzymatic mechanisms include the overexpression of efflux pump systems—most notably the AdeABC complex, where AdeB acts as the transporter—and decreased outer membrane permeability due to loss or dysregulation of the CarO porin (13, 14). These mechanisms collectively contribute to both antimicrobial resistance and virulence-associated traits like biofilm formation. While many studies have documented the presence of various resistance and virulence genes (*ompA*, *bap*, *csuA/B*), the functional relationship between gene carriage, transcriptional activity, and the resulting phenotype remains incompletely understood. Specifically, we aimed to: (i) phenotypically assess antimicrobial resistance profiles; (ii) genotypically screen for major carbapenemase and virulence-associated genes; and (iii) quantitatively evaluate the expression of the *adeB* and *carO* genes in isolates with differing meropenem MICs to elucidate their contribution to the XDR phenotype.

Materials and Methods

Bacterial Isolates and Identification

A total of forty *Acinetobacter baumannii* isolates were collected from various clinical specimens obtained from hospitalized patients at Baqiyatallah hospital. Preliminary identification of isolates was performed using standard biochemical tests routinely applied in the clinical microbiology laboratory. Molecular confirmation of species identity was subsequently carried out by polymerase chain reaction (PCR) amplification of the *16S rRNA* gene, using previously described primers and protocols (Table1). Confirmed isolates were stored at −80 °C in tryptic soy broth supplemented with glycerol until further analysis. The isolates were collected during 2025 from various clinical specimens of hospitalized patients admitted to intensive care units (ICUs), surgical wards, and internal medicine wards of Baqiyatallah Hospital in Tehran, Iran.

Ethical consideration

Prior to the commencement of the study, the complete research protocol, informed consent forms, including objectives, methodology, and data handling procedures, was critically reviewed and approved by the Ethics Committee of Tarbiat Modares University. Formal ethical approval was granted under the reference code IR.MODARES.REC.1403.049. Informed consent was obtained from all individual participants involved in the study. Participants were provided with comprehensive information regarding the study's purpose, procedures, potential risks and benefits, and their right to withdraw at any time without consequence.

Phenotypic Detection of Resistance Mechanisms

Carbapenem inactivation method (mCIM) and EDTA-carbapenem inactivation method (eCIM)

All 40 isolates met the XDR definition based on routine clinical susceptibility testing performed at the hospital’s laboratory, showing non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (including aminoglycosides, fluoroquinolones, and cephalosporins). The meropenem MICs presented here confirm high-level carbapenem resistance in the majority of isolates. For isolates with meropenem MIC = 8 µg/mL (intermediate by CLSI/EUCAST criteria), their XDR status was supported by resistance to multiple other antibiotic classes (15, 16). Phenotypic detection of carbapenemase production was performed using the modified carbapenem inactivation method (mCIM) in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, each test isolate was incubated in tryptic soy broth containing a meropenem disk, followed by placement of the disk onto a lawn culture of *Escherichia coli* ATCC 25922. After incubation, zone diameters were measured and interpreted according to CLSI criteria. To differentiate metallo-β-lactamase (MBL) producers from serine carbapenemases, the EDTA-carbapenem inactivation method (eCIM) was performed in parallel (15), using EDTA as a chelating agent. Results were interpreted based on differences in inhibition zone diameters between mCIM and eCIM assays.

Double-disk synergy test (DDST)

The double-disk synergy test (DDST) was conducted to detect the phenotypic production of extended-spectrum β-lactamases (ESBLs). Briefly, disks containing third-generation cephalosporins were placed at a defined distance from an amoxicillin–clavulanic acid disk on Mueller–Hinton agar plates inoculated with the test isolates. Enhancement or distortion of the inhibition zone toward the clavulanate-containing disk was interpreted as indicative of ESBL production.

Antibiotic susceptibility testing and MIC determination

Antimicrobial susceptibility to meropenem was evaluated by determining the minimum inhibitory concentration (MIC) using the broth microdilution method, following CLSI recommendations. Serial twofold dilutions of meropenem were prepared in cation-adjusted Mueller–Hinton broth, and bacterial suspensions were adjusted to a standardized inoculum. MIC values were recorded as the lowest concentration of meropenem that completely inhibited visible bacterial growth after incubation.

DNA extraction and conventional PCR

Genomic DNA was extracted from overnight bacterial cultures using a heat lysis (boiling) method. Cell pellets were resuspended in sterile distilled water, subjected to heat treatment at 100 °C for 10 minutes, and centrifuged to pellet cellular debris. The resulting supernatant, containing crude genomic DNA, served as the template for subsequent PCR reactions. Conventional PCR was employed to detect key genetic determinants. Targets included the intrinsic carbapenemase gene *blaOXA*-51; acquired metallo-β-lactamase genes (*blaNDM, blaVIM, blaIMP*); the presence of the ISAba1 insertion sequence upstream of *blaOXA*-51; and the virulence-associated genes *adeB* and *carO*. Amplifications were performed using gene-specific primers under previously established thermocycling conditions. Reaction products were separated by agarose gel electrophoresis and visualized under UV light.

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

For gene expression analysis, five representative isolates were selected based on their meropenem MIC values, including three isolates with the lowest MICs and two isolates with the highest MICs. Total RNA was extracted using a commercial RNA extraction kit according to the manufacturer’s instructions. Residual genomic DNA was removed by DNase I treatment. Complementary DNA (cDNA) was synthesized from purified RNA using a reverse transcription kit. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix on a StepOne Plus Real-Time PCR System. The expression levels of the *adeB* and *carO* genes were quantified and normalized against a housekeeping reference gene (*16S rRNA*). Relative gene expression levels were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method, and expression differences between high-MIC and low-MIC groups were assessed.

Statistical analysis

Statistical analysis was performed using SPSS software. An independent samples t-test was applied to compare mean Ct values and relative gene expression levels between isolates with high and low meropenem MICs. A p-value of < 0.05 was considered statistically significant.

Results

Phenotypic resistance profile

Phenotypic assays revealed widespread carbapenemase activity among the *A. baumannii* isolates. All isolates were positive by the modified carbapenem inactivation method (mCIM), confirming carbapenemase production. In contrast, the EDTA-carbapenem inactivation method (eCIM) was negative for all 40 isolates, indicating the absence of metallo-β-lactamase activity. Extended-spectrum β-lactamase (ESBL) production, as detected by the double-disk synergy test (DDST), was uncommon, identified in only 2 isolates (5%). Meropenem minimum inhibitory concentrations (MICs) ranged from 8 to 32 µg/mL. The MIC for the majority of isolates (n=16) was 16 µg/mL, while four isolates exhibited the highest MIC of 32 µg/mL, indicating high-level carbapenem resistance.

Prevalence of resistance and virulence genes

Molecular analysis confirmed species identity, with the 16S rRNA gene detected in all isolates. The intrinsic carbapenemase gene *blaOXA*-51 was present in 38 of 40 isolates (95%). Notably, the ISAba1 insertion sequence was not found upstream of *blaOXA*-51 in any positive isolate. Among acquired metallo-β-lactamase genes, *blaNDM* was identified in 8 isolates (20%), while blaVIM and *blaIMP* were not detected. Analysis of other resistance determinants revealed a high prevalence of the efflux pump gene *adeB* (35 isolates, 87.5%) and the porin gene *carO* (31 isolates, 77.5%). Importantly, all isolates lacking *adeB* were in the low-meropenem-MIC group, suggesting a link between the presence of this efflux pump gene and higher levels of carbapenem resistance.

Gene expression analysis

Quantitative real-time PCR (qRT-PCR) was used to assess *adeB* and *carO* expression in five representative isolates selected based on their meropenem MICs. Lower mean Ct values—indicative of higher gene expression—were observed in high-MIC isolates for both *adeB* (21.15 vs. 27.53) and *carO* (19.07 vs. 26.09) compared to low-MIC isolates. This pattern suggests a trend of increased expression in more resistant isolates. However, the differences were not statistically significant (*adeB*, p = 0.748; *carO*, p = 0.560), indicating that differential expression of these genes alone is insufficient to explain the observed variance in meropenem resistance.

Discussion

The unprecedented spread of antibiotic resistance, a looming pandemic, threatens the future of infection control and global public health (21). This threat is acutely embodied by the rise of carbapenem-resistant bacteria like *A. baumannii*, where treatment options are critically limited (16)—a situation exacerbated by inadequate surveillance, particularly in hospitals, which raises alarms about outbreaks of emerging XDR strains (22). This threat is exacerbated by inadequate monitoring, primarily in hospitals, raising concerns about outbreaks caused by emerging, highly resistant (XDR) strains (22, 23). To address this, we performed a comprehensive phenotypic and genotypic characterization of clinical XDR *A. baumannii* isolates. The high prevalence of the intrinsic *blaOXA*-51 gene (95%) confirms its central role. Interestingly, the absence of the ISAba1 insertion sequence upstream of *blaOXA*-51 in all isolates suggests that additional regulatory mechanisms or other resistance determinants may contribute to the observed high-level resistance. Carbapenem resistance in the examined XDR A. baumannii isolates is associated with the intrinsic blaOXA-51 gene (likely expressed at basal levels or regulated by alternative mechanisms, as ISAba1 was absent) and, in a subset of isolates, the acquired blaNDM gene. The contribution of blaOXA-51 to the overall resistance phenotype appears to be augmented by other coexisting determinants, including the high prevalence of adeB and carO. A study by Silva Rodrigues *et al* described clinical *A. baumannii* isolates co-harboring plasmid-borne blaOXA-58and blaNDM-1 highlighting the role of mobile genetic elements in rapid dissemination and high-level resistance (24). While our isolates lacked ISAba1 upstream of blaOXA-51and showed no statistically significant differences in efflux (adeB) or porin (carO) expression between high- and low-MIC groups, their study demonstrated that co-occurring carbapenemase genes on large plasmids can act as key drivers of resistance and outbreak potential. Although we observed a trend toward higher carO expression in isolates with elevated meropenem MICs, this finding contrasts with the conventional view that reduced CarO expression contributes to carbapenem resistance. The observed increase was not statistically significant and may represent a non-specific stress response or regulatory peculiarity in these clinical isolates. Given the small sample size, no firm conclusion can be drawn regarding the role of carO expression in resistance. The detection of the acquired blaNDM gene in 20% of isolates is clinically significant, highlighting horizontal transfer of a potent carbapenemase and posing challenges for both treatment and infection control. The absence of other metallo-β-lactamases (blaVIM and blaIMP) is reassuring but underscores the necessity of ongoing surveillance for emerging resistance determinants. Efflux-mediated resistance and outer membrane permeability also appear to contribute to the XDR phenotype. The high prevalence of adeB (87.5%) and carO (77.5%) supports their role in antimicrobial resistance and bacterial persistence. All *adeB*-negative isolates were in the low-meropenem-MIC group, suggesting a possible link between *adeB* carriage and increased resistance, consistent with prior reports. Gene expression analysis revealed a trend toward higher *adeB* and *carO* transcription in isolates with elevated meropenem MICs, but differences were not statistically significant. This indicates that the transcriptional levels of individual genes alone do not fully explain the observed variation in resistance. These findings highlight the multifactorial nature of carbapenem resistance, likely involving combined effects of β-lactamases, efflux pumps, porin alterations, and possibly other regulatory or post-transcriptional mechanisms. The limited sample size for expression analysis (n = 5) may have constrained statistical detection of subtle, biologically meaningful differences. Overall, these results emphasize the complexity of carbapenem resistance in XDR A. baumannii, reinforcing that both intrinsic and acquired determinants, together with multiple resistance mechanisms, drive the observed phenotype.

Conclusion

In conclusion, our findings establish that carbapenem resistance in XDR *A. baumannii* isolates is principally driven by the intrinsic *blaOXA*-51 gene, augmented in a subset of strains by the acquired *blaNDM*. The near-ubiquitous presence of *adeB* and *carO* underscores their status as core resistance determinants. However, the lack of a statistically significant correlation between their expression and the resistance phenotype demonstrates that high-level resistance is not dictated by single genetic determinants but arises from a complex interplay within a broader network. To fully decipher this network, future work must employ integrated whole-genome sequencing and transcriptomic profiling across larger clinical cohorts. Such comprehensive analyses are essential to unravel the precise molecular mechanisms driving resistance, ultimately informing targeted therapeutic and infection control strategies. Consequently, enhanced surveillance for strains carrying and potentially mobilizing *blaOXA*-51, alongside those bearing *blaNDM*, is crucial for preempting and controlling hospital outbreaks.

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During manuscript preparation, DeepSeek (DeepSeek AI) was utilized exclusively for language polishing, specifically for grammar and spelling review. Its use was strictly limited to this purpose; it was not involved in designing the narrative structure, analyzing data, or generating scientific content.

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