ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2026;12(1):53-60 10.52547/iem.12.1.53

Molecular Epidemiology of *Acinetobacter baumannii* Isolates Using Single-Locus Sequence Typing of $bla_{\text{OXA-51-like}}$ and *ampC* Genes as a Cost-Effective Method

ARTICLE INFO

Article Type
Original Article

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How to cite this article
Firoozeh F., Hoseini Z., *Mohammadzadeh M.,* Badmasti F., Zibaei M., Zibaei S. Molecular Epidemiology of Acinetobacter baumannii Isolates Using Single-Locus Sequence Typing of blaOXA-51-like and ampC Genes as a Cost-Effective Method. Infection Epidemiology and Microbiology. 2026;12(1): 53-60.

Article History
Received: September 27, 2025
Accepted: April 28, 2026
Published: May 26, 2026

ABSTRACT

Background: *Acinetobacter baumannii* is a crisis-causing opportunistic pathogen with a high capacity for clonal spread. $bla_{\text{OXA-51-like}}$ and *ampC* sequence-based typing (SBT) is a cost-effective and reliable technique to identify global and widespread lineages of *A. baumannii* strains. This research aimed to study circulating *A. baumannii* clones using single-locus sequence typing (SLST) as a reliable and cost-effective method.

Materials & Methods: A total of 119 *A. baumannii* clinical isolates were collected from hospital inpatients from February to September 2024. The antibiotic resistance profile of *A. baumannii* isolates was determined, and genotyping was performed using SBT of $bla_{\text{OXA-51-like}}$ and *ampC* genes.

Findings: All *A. baumannii* isolates were specified as multidrug-resistant (MDR), and 78.1% were identified as carbapenem resistant. Resistance to colistin was observed in 5% of isolates with a minimum inhibitory concentration (MIC) of ≥128 µg/mL. SBT of the $bla_{\text{OXA-51-like}}$ gene revealed four various $bla_{\text{OXA-51-like}}$ allele variants: including $bla_{\text{OXA-69}}$ (n=47, 39.5%), $bla_{\text{OXA-64}}$ (n=21, 17.6%), $bla_{\text{OXA-383}}$ (n=44, 37%), and $bla_{\text{OXA-441}}$ (n=7, 5.9%). SBT of the *ampC* specified two alleles, including *ampC-1* (n=47, 39.5%) and *ampC-25* (n=21, 17.6%), and 51 (42.9%) isolates showed an undetermined allele variant of the *ampC* gene. $bla_{\text{OXA-69}}$/*ampC-1* and $bla_{\text{OXA-64}}$/ *ampC-25* pertained to sequence type 1/ clonal complex 1 (ST1/CC1) and ST25/CC25, respectively.

Conclusion: SBT of the $bla_{\text{OXA-51-like}}$ and *ampC* genes revealed a relatively high prevalence of the international ST1/CC1 clone in MDR *A. baumannii* isolates in our region. However, the $bla_{\text{OXA-383}}$ allele variant, which could possibly be associated with endemic clones, also had an approximately significant frequency.

Keywords: *Acinetobacter baumannii*, Molecular epidemiology, Genotyping, Multidrug-resistance.

CITATION LINKS

[1] You Q, et al. Local characteristics of... [2] Li T, et al. Comparing core-genome... [3] Afzali H, et al. Characterization of ... [4] Chukamnerd A, et al. Whole-genome analysis of... [5] Wang M, et al. Clinical outcomes and... [6] Firoozeh F, Omidi M, Saffari M, Sedaghat H, Zibaei M. Molecular... [7] Piran A, Shahcheraghi F, Solgi H, Rohani M, Badmasti F. A reliable... [8] Diancourt L, et al. The population... [9] Bartual SG, et al. Development of... [10] Pournaras S, et al. Single-locus... [11] Hamouda A, Evans BA, Towner KJ, Amyes SG. Characterization of... [12] Hamed SM, et al. Acinetobacter baumannii ... [13] Karah N, et al. Novel aminoglycoside... [14] Bakhshi F, et al. Molecular detection of ... [15] Clinical and Laboratory Standards Institute... [16] Firoozeh F, et al. Characterization... [17] Magiorakos AP, et al. Multidrug-resistant,... [18] Abhari SS, Badmasti F, Modiri L, Aslani MM, Asmar M. Circulation of... [19] Perez F, Hujer AM, et al. Global challenge of... [20] Singh S, Singh S, Trivedi M, Dwivedi M. An insight... [21] Firoozeh F, et al. Clonal relatedness of... [22] Alcántar-Curiel MD, et al. Carbapenem-resistant ... [23] Bagheri-Josheghani S, et al. Quinolone-resistant... [24] Villalón P, et al. Dynamics of... [25] Sahl JW, et al. Phylogenetic and... [26] Da Silva KE, et al. A high... [27] Villalón P, et al. Endemic... [28] Shelenkov A, Akimkin V, Mikhaylova Y. International... [29] Nazari M, et al.. Emergence of... [30] Karah N, Jolley KA, Hall RM, Uhlin BE. Database for... [31] Gaiarsa S, et al. Comparative analysis of... [32] Karah N, Giske CG, Sundsfjord A, Samuelsen Ø. A diversity of... [33] Stietz MS, et al. Acinetobacter baumannii...

Introduction

Acinetobacter baumannii has been recognized in recent decades as a notoriously drug-resistant nosocomial pathogen responsible for severe infections, particularly in intensive care unit (ICU) patients [1-3]. Hospital outbreaks due to multidrug-resistant (MDR) *A. baumannii* strains are increasingly being recorded worldwide, most of which are also carbapenem-resistant [4]. According to the results obtained of previous investigations, the strains associated with the outbreak belong to different clonal lineages, mainly international clones (ICs) I - III [5].

To find sources and monitor disease outbreaks and to demonstrate clonal relatedness of strains, various genotyping methods with varying levels of specificity are available [6]. The most widely applied genotyping methods are as follow: multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), repetitive sequence-based polymerase chain reaction (rep-PCR), randomly amplified polymorphic DNA (RAPD) analysis, multiple loci variable number tandem repeats (VNTRs) analysis (MLVA), amplified fragment length polymorphism (AFLP), 3-locus sequence typing (3-LST), and other techniques [6, 7]. Among the various methods, the reference method and "gold standard" is MLST, of which there are two different schemes, including Oxford and Pasteur [8, 9]. Each of the aforementioned schemes is based on the sequences of multiple housekeeping genes and is commonly applied for epidemiological investigations at global and local scales [10]. Among sequence-based typing (SBT) methods, SBT of the $bla_{\text{OXA-51-like}}$ and *ampC* genes has been documented as a typing method with similar discriminatory power to Oxford and Pasteur's MLST [10, 11]. In a PCR-based typing scheme in a study, the $bla_{OXA\text{-}51\text{-like}}$ gene was used as one of three loci, which could assign *A. baumannii* isolates to sequence groups (SGs), thereby demonstrating correlation between SGs and the major epidemic lineage within the species [12]. Considering that particular *ampC* alleles are associated with specific clones of *A. baumannii,* analysis of the *ampC* locus could also be an appropriate method for molecular epidemiological studies of *A. baumannii* [13].

Objectives: Since typing of epidemic clones could provide useful information about their emergence and spread, and given the relatively limited information in this field in our region, the present study aimed to investigate circulating *A. baumannii* clones isolated from clinical specimens using single-locus sequence typing (SLST) as a reliable and cost-effective method.

Materials and Methods

Recognition of *A. baumannii* isolates: In the current cross-sectional study, 119 *A. baumannii* clinical isolates were collected from patients hospitalized in Karaj hospitals in Alborz province from February to September 2024. Clinical samples were as follows: urine, endotracheal tube aspirate, sputum, and wound pus. The implementation of this study was authorized by Ethics Committee of Alborz University of Medical Sciences (IR.ABZUMS.REC.1401.354), and written informed consent was obtained from all study participants.

All clinical isolates were diagnosed as *A. baumannii* using standardized biochemical tests, including oxidase, catalase, oxidative-fermentative (OF), motility, indole, methyl red (MR), Voges-Proskauer (VP), triple sugar iron agar (TSI), and growth at 42 °C, and confirmed using PCR for *rpoB*, $bla_{\text{OXA-51}}$, and gluconolactonase genes [14].

Antibiotic sensitivity testing: Disk diffusion test was used to determine the susceptibility of *A. baumannii* clinical isolates to different antibiotics in accordance with the guidelines presented by the Clinical and Laboratory Standards Institute (CLSI) and the European

Committee on Antimicrobial Susceptibility Testing (EUCAST) [15,16]. Antibiotic-containing discs included doxycycline, ciprofloxacin, gentamicin, ampicillin-sulbactam, ceftriaxone, ceftazidime, and trimethoprim/sulfamethoxazole (Mast, UK). Resistance to imipenem and colistin was determined using E-test (Liofilchem, Italy). *Escherichia coli* ATCC 25922 reference strain was used as a quality control for the experiment, and *A. baumannii* isolates were considered MDR if they were resistant to at least three antimicrobial classes [17].

Single-locus molecular typing of *bla*$_{\text{OXA-51-like}}$ and *ampC* genes: To extract DNA from *A. baumannii* isolates, the boiling method was performed on two to three pure colonies of bacteria in distilled water for 10 min [14]. Amplification of the *bla*$_{\text{OXA-51-like}}$ and *ampC* genes was carried out using PCR to detect a 988 bp amplicon (with primer sequences F: CTAATAATTGATCTACTCAAGTTAC and R: GAATACTCCATTTGAACCARTGG) and a 1259 bp amplicon (with primer sequences F: AGGAGCTAATCATGCGATT and R: CAAGCTTAGGATATGTTTGG), respectively [18]. The amplification programs were as follows: a pre-denaturation step at 95 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55.1 °C for *bla*$_{\text{OXA-51-like}}$ and 56 °C for *ampC* gene for 45 s, and extension at 72 °C for 1 min; ending with a final elongation step at 72 ºC for 10 min [18].

Determination of clonal complexes (CCs) and sequence types (STs): The amplicons of the *bla*$_{\text{OXA-51-like}}$ and *ampC* genes were purified and sequenced using an ABI 3730xl DNA analyzer (Life Technologies). The resulting sequences were compared against reference databases using the BLAST sequence comparison tool. To determine alleles, each unique nucleotide sequence was assigned a distinct allele number, and new alleles were defined by ≥1 nucleotide substitution. Identification of known STs were performed based on the allele number of the single locus and searching for related ST in the curated database. Clonal complex assignments were inferred based on proven correlations between specific *bla*$_{\text{OXA-51-like}}$ or *ampC* gene alleles, and known CCs.

Findings

Detection of *A. baumannii*: In this study, 119 strains were confirmed as *A. baumannii*. They were isolated from 54 (45.4%) and 65 (54.6%) male and female patients with an average age of 48.02 years, respectively. The clinical isolates were collected from sputum (n=41, 34.5%), urine (n=19, 15.9%), endotracheal tube aspirate (n=30, 25.2%), and wound pus (n=29, 24.4%) (Table 1).

Antibiotic resistance patterns: The antibiotic sensitivity testing of *A. baumannii* isolates showed that all 119 (100%) *A. baumannii* isolates were MDR, and their highest antibiotic resistance rates were against ciprofloxacin (n=113, 94.9%), gentamicin (n=111, 93.3%),

Table1) Summary of patients› demographic data

Characteristic	Quantity N (%)
Gender	
Male	54 (45.4)
Female	65 (54.6)
Age (years)	
≤ 50	62 (52.1)
> 50	57 (47.9)
Mean ± SD[a]	48.02
Isolation source	
Sputum	41 (34.5)
Endotracheal tube aspirate	30 (25.2)
Wound pus	29 (24.4)
Urine	19 (15.9)
Isolation ward	
ICU[b]	56 (47.1)
Surgery	19 (16.0)
Infectious ward	39 (32.7)
Emergency	5 (4.2)
Length of hospital stay (days)	
< 7	88 (73.9)
7-10	20 (16.8)
> 10	11 (9.3)
Mean ± SD	5.98

[a]SD: Standard deviation
[b]ICU: Intensive care unit

Table 2) Antibiotic sensitivity profile of 119 *A. baumannii* isolates

Antibiotic	Sensitive, N (%)	Intermediate, N (%)	Resistant, N (%)
Imipenem	0 (0)	26 (21.8)	93 (78.1)
Ampicillin-sulbactam	86 (72.3)	21 (17.6)	12 (10.1)
Ceftazidime	0 (0)	18 (15.1)	101 (84.9)
Ciprofloxacin	0 (0)	6 (5.1)	113 (94.9)
Gentamicin	3 (2.5)	5 (4.2)	111 (93.3)
Doxycycline	83 (69.7)	2 (1.7)	34 (28.6)
Trimethoprim/sulfamethoxazole	7 (5.9)	17 (14.3)	95 (79.8)

Table 3) Sequence typing of $bla_{\text{OXA-51-like}}$ and *ampC* genes. $bla_{\text{OXA-51-like}}$ and *ampC* allele variants, sequence types (STs), and clonal complexes (CCs) of 119 *A. baumannii* isolates

Isolate, (n)	Source	$bla_{\text{OXA-51-like}}$ Allele	*ampC* Allele Number	Sequence Type	Clonal Complex
19	Urine	$bla_{\text{OXA-69}}$	*ampC-1*	ST1	CC1
25	Endotracheal tube	$bla_{\text{OXA-69}}$	*ampC-1*	ST1	CC1
3	Sputum	$bla_{\text{OXA-69}}$	*ampC-1*	ST1	CC1
5	Endotracheal tube	$bla_{\text{OXA-64}}$	*ampC-25*	ST25	CC25
8	Sputum	$bla_{\text{OXA-64}}$	*ampC-25*	ST25	CC25
8	Wound pus	$bla_{\text{OXA-64}}$	*ampC-25*	ST25	CC25
27	Sputum	$bla_{\text{OXA-383}}$	Not detectable	Not available	Not available
17	Wound pus	$bla_{\text{OXA-383}}$	Not detectable	Not available	Not available
3	Sputum	$bla_{\text{OXA-441}}$	Not detectable	Not available	Not available
4	Wound pus	$bla_{\text{OXA-441}}$	Not detectable	Not available	Not available

ceftazidime (n=101, 84.9%), trimethoprim/sulphamethoxazole (n=95, 79.8%), and imipenem [minimum inhibitory concentration (MIC) ≥ 16 µg/mL] (n=93, 78.1%), respectively (Table 2). The lowest resistance was observed to ampicillin-sulbactam and doxycycline, with 86 (72.3%) and 83 (69.7%) *A. baumannii* isolates being sensitive to these antibiotics, respectively. The E-test results showed that six (5.0%) *A. baumannii* isolates were colistin -resistant strains with MIC ≥ 128 µg/mL.

Sequence typing of $bla_{\text{OXA-51-like}}$ and *ampC* genes: The SBT results of $bla_{\text{OXA-51-like}}$ revealed four distinct $bla_{\text{OXA-51-like}}$ allele variants including: $bla_{\text{OXA-69}}$ (n=47, 39.5%), $bla_{\text{OXA-64}}$ (n=21, 17.6%), $bla_{\text{OXA-383}}$ (n=44, 37%), and $bla_{\text{OXA-441}}$ (n=7, 5.9%). SBT of the *ampC* gene revealed that 47 (39.5%) isolates belonged to *ampC-1*, and 21 (17.6%) isolates belonged to *ampC-25,* while 51 (42.9%) isolates were not detectable. Likewise, $bla_{\text{OXA-69}}$ /*ampC-1* belonged to ST1/CC1, and $bla_{\text{OXA-64}}$ / *ampC-25* pertained to ST25/CC25 (Table 3).

Discussion

A. baumannii is currently recognized as a global health problem due to the spread of MDR-ICs worldwide [19]. The rapid spread of the aforementioned clones necessitates the investigation of the epidemic evolution of *A.*

baumannii strains through the study of their clonal lineage [10]. Recent studies have shown a correlation between MDR *A. baumannii* clinical isolates and the prevalence of specific lineages [20]. In this study, along with resistance profile evaluation, PCR amplification followed by sequencing of the $bla_{\text{OXA-51-like}}$ gene was used as one of the simplest and most cost-effective approaches for typing *A. baumannii* strains.

Resistance and susceptibility profiles of the strains indicated that all *A. baumannii* isolates studied were MDR. Also, 78.1% of the isolates were carbapenem-resistant *A. baumannii* (CRAB) strains, which is consistent with the results of other studies in this field [16, 21-23]. However, compared to our previous studies, the rate of resistance to carbapenems was relatively lower, which could be related to differences in isolation sources [16, 21-23].

A remarkable finding in this study was that compared to our previous survey, in which no colistin-resistant *A. baumannii* strains were identified, the rate of colistin resistance in CRAB strains was relatively high (5.0%) [14, 16, 21]. Given that colistin-resistant *A. baumannii* strains were isolated from ICU and surgical ward, there may be a possibility of outbreaks of these dangerous strains in patients hospitalized in these wards.

The SBT results of the $bla_{\text{OXA-51-like}}$ gene of *A. baumannii* strains showed the acquisition of common and international lineages CC1 and CC25. Reports of CC1 and CC25 clones have been recorded from different parts of the world in *A. baumannii* isolates from clinical and non-clinical samples, which may indicate the adaptation and persistence of the aforementioned clones in different climates [24-26].

The SBT results of the $bla_{\text{OXA-51-like}}$ gene also revealed that the $bla_{\text{OXA-69}}$ allele variant, which belongs to ST1/CC1, had the highest prevalence among *A. baumannii* clinical isolates. ST1, as an IC, has a global distribution and has been documented from all over the world [7, 27]. However, in contrast to the present study, another study in our region recorded a much lower prevalence of ST1 [7, 27]. The difference in the prevalence of the mentioned clone could have occurred over time.

Notably, ST1 belongs to the IC-I, which, in addition to its wide global distribution, is associated with hospital outbreaks caused by *A. baumannii* and commonly carries a significant set of antimicrobial resistance determinants [28]. Since the majority of *A. baumannii* strains in the present study belonged to IC1 with high antibiotic resistance, the potential risk of further spread of these strains in our region is of great concern. In addition, the findings indicated that a significant percentage of isolates (37.0%) belonged to the $bla_{\text{OXA-383}}$ allele variant, and a number of isolates (5.9%) were also identified as $bla_{\text{OXA-441.}}$ Until now, no reports of the mentioned allele variants have been found in other studies in the world. It seems that some clones emerge in certain climatic conditions and potential reservoirs.

Based on the findings obtained from SBT analysis of the *ampC* locus, a large number of the studied *A. baumannii* isolates were related to the *ampC-1* allele (CC1), and some were also identified as the *ampC-25* allele (CC25), while a relatively large number of isolates were also not detected. These findings are consistent with the results of other studies conducted in our region and elsewhere [21, 29, 30]. Likewise, CC1, which is known as the globally distributed *A. baumannii* clonal complex, has been isolated from different parts of the world for more than two decades [25,31]. Also, *A. baumannii* strains classified as ST25, were the cause of epidemics in European countries and have been isolated endemically or sporadically in South America and Asia [25, 32, 33].

Conclusion

The final conclusion is that, according to the results of sequence typing of $bla_{\text{OXA-51-like}}$ and *ampC* genes, there is a relatively high prevalence of the international ST1/CC1 clone in MDR *A. baumannii* clinical isolates in our region. However, $bla_{\text{OXA-383}}$ allele variant, which could possibly be associated with endemic clones, also has an approximately significant frequency.

Acknowledgments

The authors would like to express their deepest gratitude for the technical assistance of the Microbiology Laboratory, Department of Microbiology, Alborz University of Medical Sciences and the Bacteriology Laboratory, Pasteur Institute of Iran.

Ethical permissions: All methods were carried out in accordance with the relevant guidelines and regulations of Ethics Clearance Committee of Alborz University of Medical Sciences (ethic code: IR.ABZUMS.REC.1401.354).

Authors' contributions: Conceptualization: Farzaneh Firoozeh, Farzad Badmasti, and Zahra Hosseini; Data curation: Farzaneh Firoozeh, Zahra Hosseini, and Farzad Badmasti; Formal analysis and investigation: Mohammad Mohammadzadeh; Funding acquisition: Farzaneh Firoozeh; Methodology: Farzaneh Firoozeh, Zahra Hosseini, Mohammad Mohammadzadeh, and Farzad Badmasti; Project administration: Farzaneh Firoozeh; Supervision: Farzaneh Firoozeh and Farzad Badmasti; Writing of the original draft: Farzaneh Firoozeh, Sepeta Zibaei, and Mohammad Zibaei; Writing, reviewing, and editing: Farzaneh Firoozeh and Mohammad Zibaei.

Conflicts of interest: The authors declare that there are no conflicts of interest.

Fundings: Our institute had not role in study design, data collection and analysis, publication decision, manuscript preparation, and financial support.

Consent to participate: Written informed consents were obtained from all participants.

Competing interests: The authors declare that they have no conflicts of interests.

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