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Correlation Between IS*Aba1* Upstream ampC Gene and Resistance to Cefotaxime in *Acinetobacter baumannii*: A Serious Threat to Nosocomial Infections

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Abstract

Background: Infections due to *Acinetobacter baumannii* have become a significant challenge in modern healthcare systems. The global upsurge of multidrug resistance in *A. baumannii* has created widespread problems in the treatment of patients.
Objectives: We examined the prevalence ISAmpC and its correlation with cefotaxime resistance.
Materials and Methods: Standard biochemical tests were used to identify isolates. Genomic species of the genus *Acinetobacter* were confirmed by Amplified Ribosomal DNA Restriction Analysis (ARDRA). The susceptibility of 50 *A. baumannii* isolates to a variety of antimicrobial agents was determined using the disk diffusion method and E-test strips. PCR was used to investigate the connection of insertion sequences and the ampC gene. Clonal relatedness was determined by Repetitive Extragenic Palindromic PCR.
Results: IS*Aba1* located upstream of bla_{ampC} was found in 24 (48%) of the *A. baumannii* isolates. In all of the studied isolates that had ISAmpC, the MIC for cefotaxime was 64 - 256 μg/mL. Based on the REP-PCR patterns among the resistant isolates, the highest number of ISAmpC positive isolates belonged to type B (n = 19) and type C (n = 12).
Conclusions: IS*Aba1* has become an important factor in *A. baumannii*'s resistance to cefotaxime.

Keywords: IS*Aba1*, ampC, Nosocomial Infection, *Acinetobacter baumannii*

1. Background

Members of the genus *Acinetobacter* are aerobic, non-fermenting, Gram-negative bacilli, which are responsible for a wide spectrum of infections in immunocompromised hosts (1). The genus *Acinetobacter* currently contains up to 32 described named and unnamed (genomic) species. *Acinetobacter baumannii*, genomic species 3, and 13TU, three of the most clinically relevant species, are genetically and phenotypically very similar to an environmental species, *Acinetobacter calcoaceticus*, and are therefore grouped together in the so-called *A. calcoaceticus A. baumannii* (Acb) complex. Numerous reports have commented on Acinetobacter-related hospital infections and outbreaks. Strains belonging to genomic species 2 (*A. baumannii*), genomic species 3, and 13TU have been found to be most frequently associated with hospital -acquired infections and epidemic outbreaks. *Acinetobacter baumannii* has emerged over the last decade as a significant opportunistic pathogen. Although it is generally associated with benign colonization of hospitalized patients, it is responsible for about 10% of nosocomial infections in intensive care unit (ICU) patients, causing a wide range of infections such as bacteremia, nosocomial pneumonia, urinary tract infections, secondary meningitis, and burn and wound infections. In acinetobacter-associated nosocomial infections, the major problem encountered by ICU clinicians relates to the readily transferable antimicrobial resistance expressed by this organism (2). It is often multi-resistant to antibiotics, meaning that therapy and infection control are complicated (3). Antimicrobial treatment for such infections, particularly for multi-drug resistant (MDR) *Acinetobacter* spp., which is resistant to multiple antibiotics including aminoglycosides, extended-spectrum cephalosporins, carbapenems, and fluoroquinolones, is extremely difficult. Beta-lactam and extended-spectrum cephalosporin antibiotics are effective choices for the treatment of infections caused by MDR *A. baumannii* isolates.

Generally, resistance to beta-lactams is related to beta-lactamase production, modification of penicillin-binding proteins, and the increased activity of efflux pumps and reduced penetration across the outer membrane (4, 5). In *A. baumannii*, resistance to beta-lactams is related mostly to the expression of beta-lactamases. Class C beta-lactamases are chromosomally encoded cepha-

losporinases (AmpC), which are naturally produced by all strains of *A. baumannii* (6). These enzymes hydrolyze penicillins and cephalosporins, but not cefepime or carbapenems. AmpC can be upregulated as a consequence of the upstream insertion of an IS*Aba1* sequence, which provides an efficient promoter. Insertion of this element upstream of the bla_{ampC} gene is thought to serve as a "moving switch" to turn on those genes with which it is juxtaposed. Resistance to cephalosporins in *A. baumannii* is related primarily to the high-level expression of the bla_{ampC} beta-lactamase gene. An insertion sequence (IS) element upstream of the bla_{ampC} gene has the potential to cause over-expression of AmpC (7, 8).

2. Objectives

The present study focuses on the role of the insertion of IS*Aba1* upstream of the bla_{ampC} gene and its correlation with cefotaxime resistance.

3. Materials and Methods

3.1. Bacterial Strains and Culture Conditions

Between January 2013 and June 2014 *Acinetobacter baumannii* isolates were recovered from clinical specimens in intensive care units in hospitals in Tehran, Iran. Isolates were recovered from clinical samples after culturing on MacConkey agar and incubating for 24 hours at 37°C. Non-lactose fermenting bacteria (colorless or slightly beige) were sub-cultured and incubated for an additional 24 hours. Clinical isolates were confirmed using standard bacteriological methods performed at the medical microbiology laboratory at the Tabriz University of Medical Sciences. The following tests were applied: Gram stain (Gram-negative coccobacilli), oxidase test (negative), motility (nonmotile), catalase test (positive), citrate test (positive), growth at 37°C and 44°C, and production of acid from glucose in O/F medium (9). Genomic species of *Acinetobacter* spp. were confirmed by Amplified Ribosomal DNA Restriction Analysis (ARDRA). PCR products of 16S rRNA were digested with AluI, MboI, and HhaI restriction enzymes (Fermentas, St. Leon-Rot, Germany) (10, 11). The ARDRA method used was described previously (12). Briefly, strains were grown on Mueller-Hinton agar. A 1-mL loopful of colony growth was suspended in 300 mL of distilled water, boiled for 10 minutes, agitated thoroughly, and centrifuged briefly in a microcentrifuge. With filter-protected tips, 5 μL of the supernatant was added to 45-mL aliquots of a PCR mix containing 1.25 U of Taq polymerase (Fermentas, UK), 100 mM (each) deoxynucleoside triphosphates, and 0.2 mM (each) primer in reaction buffer (1.5 mM $MgCl_2$ and 50 mM KCl in 10 mM Tris-HCl, pH 8.3), overlaid with 40 mL of mineral oil. After initial denaturation at 95°C for 5 minutes, the reaction mixture was run through 35 cycles of denaturation at 95°C for 45 seconds, annealing at 508°C for 45 seconds, and extension at 72°C for 1 minute. Finally, a 7- minutesextension period was carried out at 72°C (10, 11).

3.2. Antimicrobial Susceptibility Testing

The disk diffusion method was used to assess susceptibility to the following antimicrobial agents: ampicillin-sulbactam (10/10 μg), piperacillin-tazobactam (100/10 μg), ticarcillin (75 μg), cefotaxime (30 μg), ceftazidime (30 μg), and cefepime (30 μg) (MAST, Merseyside, UK). Moreover, the MIC value for cefotaxime was determined using E-test strips (Liofilcheme, Italy) on Mueller-Hinton agar (Liofilcheme, Italy). E-test papers containing different concentrations of each antibiotic were placed on Muller-Hinton agar plates that had been inoculated with bacterial suspensions corresponding to a 0.5 McFarland turbidity standard. The plates were then incubated at 37°C for 24 hours. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls in each susceptibility determination (13).

3.3. Detection of ISAba1 by PCR

All of the *A. baumannii* isolates were grown for 18 hours at 37°C on MacConkey agar and DNA was extracted using the SDS-Proteinase K phenol chloroform method, as previously described. Briefly, 4 - 5 fresh colonies were resuspended in 300 μL of TE buffer, SDS (1%), and proteinase K (10 μg/mL), and incubated at 40°C for 3 hours followed by phenol-chloroform extraction and ethanol precipitation. Finally, the DNA was dissolved in distilled water and the quality and concentration of the DNA were checked using electrophoresis on a 1% agarose gel and a spectrometer, respectively (14). PCR was carried out in a thermocycler. Reaction mixes contained 20 pmol of each primer (the sequences of the primers are shown in Table 1), 800 MM dNTPs, 2.5 U Taq DNA polymerase, and 2 μL of the chromosomal DNA (Qiagen) in a final volume of 50 μL. Initial denaturation (95°C for 5 minutes) was followed by 35 cycles of amplification. Each cycle consisted of 95°C for 45 seconds, 56°C for 45 seconds, and 72°C for 3 minutes. A final extension step (72°C for 5 minutes) completed the amplification. The amplified product was resolved by electrophoresis in 1.5% (wt/vol) agarose gel containing ethidium bromide (50 mg/mL) (15).

Table 1. Primers Used in This Study

Genes	Forward (5'-3')	Reverse (5'-3')	Annealing Temperature, °C
AmpC	ACAGAGGAGCTAATCATGCG	GTTCTTTTAAACCATATACC	49
IS/AmpC	GACCTGCAAAGAAGCGCTGC	GTTCTTTTAAACCATATACC	45
IS*Aba1*	CACGAATGCAGAAGTTG	CGACGAATACTATGACAC	56

3.4. Detection of ampC by PCR

DNA extraction was carried out using the Cetyl trimethylammonium bromide (CTAB) method (14). Amplification of the AmpC coding sequence was performed both with and without the IS*Aba1* promoter sequences. The primers used for PCR amplification are listed in Table 1. PCR reactions were carried out with a 50-mL volume of a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 2.0 mM magnesium chloride, 200 mM deoxynucleotide triphosphate, 50 pmol of each primer, 500 ng of the chromosomal DNA, and 2.5 U of Taq polymerase. Amplification reactions were submitted to the following program: initial denaturation (4 minutes at 94°C), followed by 30 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 50°C), and extension (2 minutes at 72°C), with a single extension of 10 minutes at 72°C. The amplified product was resolved by electrophoresis in a 1.5% (wt/vol) agarose gel containing ethidium bromide (50 mg/mL) (15).

3.5. Repetitive Extragenic Palindromic PCR (REP-PCR) Analysis

The primer pairs REP1, 5'-IIIGCGCCGICATCAGGC-3', where I = inosine and REP2, 5'-ACGTCTTATCAGGCCTAC-3' were used to amplify the putative REP-like elements in the genomic bacterial chromosomes. Strains belonging to the same type (DNA group) showed identical profiles or highly similar profiles (up to 2 bands difference). REP-PCR was carried out in a final volume of 25 μL, containing 2.5 μL 10 X PCR buffer, 0.5 μL of dNTP Mix (10 mol), 5 μL $MgCl_2$, 25 pM REP-primer REP1, 25 pM REP-primer REP2, 2 U of Taq DNA polymerase, 3 μL extracted template DNA, and 16.1 μL distilled water. Aliquots (12 μL) of each sample were subjected to electrophoresis on a 1.2% agarose gel. Amplified products were detected by staining with ethidium bromide and photographed with Polaroid type 665 film. To group isolates for photographic documentation, the REP-PCR fingerprints of strains were exposed to UV light, photographed, and compared by visual inspection. Amplification was performed with an initial denaturation at 95°C for 10 minutes, followed by 30 cycles of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 2 minutes. The final extension was done at 72°C for 16 minutes (16).

3.6. Statistical Analysis

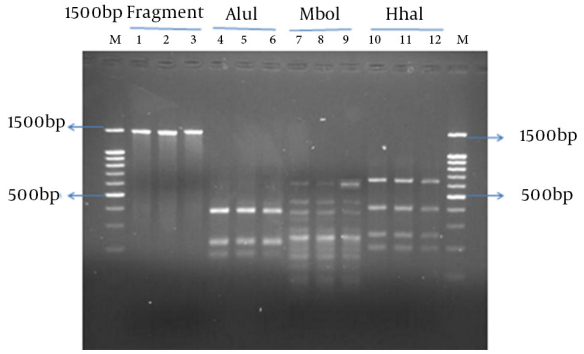
Analyses were performed by SPSS software version 16. A Chi-square test was used to calculate the association between cefotaxime resistance and ISAmpC. The significance level was defined as $P < 0.01$.

4. Results

Among the *Acinetobacter* spp. isolates from the hospitals in Tehran, Iran, 50 isolates showed the same ARDRA profiles as *A. baumannii* ATCC 19606 (Figure 1). In our study, *Acinetobacter* isolates were recovered from 50 patients. The male to female ratio was 3.1:1 (38 males and 12 females). The age range of the patients was 10 to 89 years. Antimicrobial susceptibility testing showed that, respectively, 96%, 88%, 100%, 100%, 64%, and 98% of isolates were resistant to ampicillin-sulbactam, cefotaxime, ceftazidim, cefepime, piperacillin-tazobactam, and ticarcillin. In addition, 42% of the isolates showed an MIC value of 256 μg/mL for cefotaxime. PCR was then performed with template DNA from all strains. Following this reaction, some strains yielded an amplicon of ~356 bp, but other isolates gave a ~1500 bp amplicon. These results suggest an insertion of IS*Aba1* located upstream of the ampC gene in the isolates.

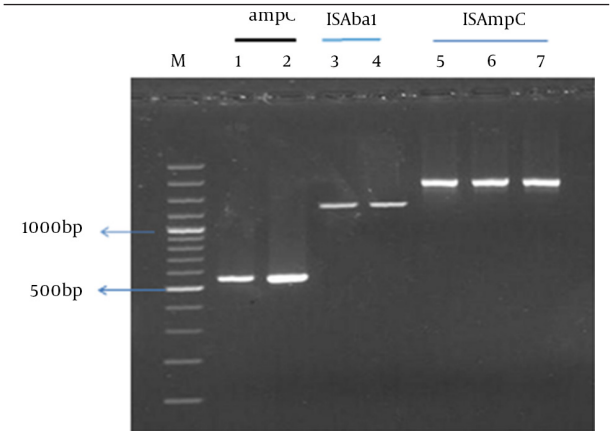
IS*Aba1* located upstream of bla_{ampC} was observed in 24 (48%) of the *A. baumannii* isolates (Figure 2). In all of the studied isolates that had ISAmpC, the MIC for cefotaxime was 64 - 256 μg/mL. Resistant isolates were grouped into five clusters (A, B, C, D, and E) based on their REP-PCR patterns (Table 2) (Figure 3). Based on REP-PCR patterns among the resistant isolates, the highest ISAmpC positive isolates belonged to type B (n = 19) and type C (n = 12).

Figure 1. Overview of the Patterns Obtained After Restriction of the Amplified 16S rRNA Gene With Three Different Enzymes



Lane M, molecular size marker (100-bp ladder); Lanes 1, 4, 7, and 10, *A. baumannii* ATCC 19606 (positive control). Other lanes are positive clinical *A. baumannii* isolates.

Figure 2. PCR amplification of bla_{ampC}, IS*Aba1*, and ampC Genes in *A. baumannii* Isolates



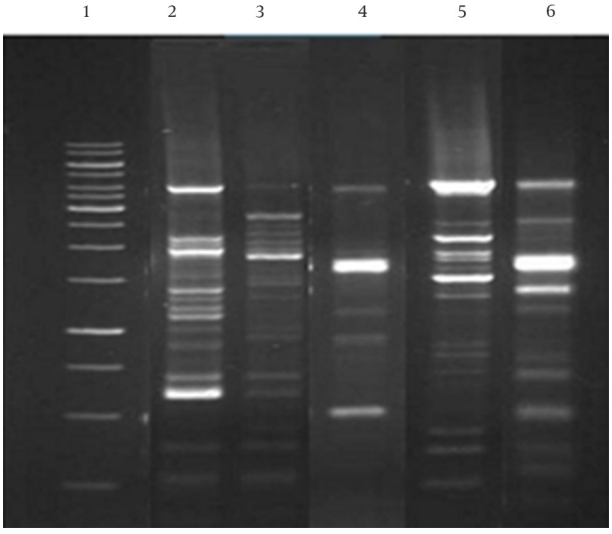
Lane M, 100 bp plus ladder; Lanes 1 and 2, positive clinical *A. baumannii* isolates for bla_{ampC} gene; Lanes 3 and 4, positive clinical *A. baumannii* isolates for IS*Aba1*; Lanes 5, 6, and 7, positive clinical *A. baumannii* isolates for ISAmpC.

Table 2. Identification of IS/ampC Genes Among Different Clones of the Studied Isolates

REP-Types	IS*Aba1*/ampC[a]	CTX MIC (Range)
A (n = 7)	2	> 64
B (n = 19)	12	> 128
C (n = 12)	4	> 128
D (n = 8)	4	> 256
E (n=4)	2	> 256

Abbreviations: CTX, cefotaxime; MIC, minimum inhibitory concentration.
[a]Presence of IS

Figure 3. REP-PCR Pattern of Resistant *A. baumannii* From Clinical Isolates



Lane 1, DNA size marker 1 kbp; Lane 2, genotype A; Lane 3, genotype B; Lane 4, type C; Lane 5, type D; Lane 6, type E.

5. Discussion

Resistance patterns among nosocomial bacterial pathogens may vary in hospitals in different parts of the world. *Acinetobacter baumannii* is one of the most important nosocomial pathogens, particularly in ICUs, and causes various types of infections, including pneumonia, urinary tract infections, wound infections, bacteremia, and meningitis. There are increasing reports of multidrug-resistant *A. baumannii* (MDR-AB) outbreaks in clinical settings worldwide (2, 3). Beta-lactam drugs compose more than 50% of all antibiotics used in clinics (17) yet the therapeutic benefits of this group of drugs is decreasing because of the growing prevalence of bacterial resistance mechanisms. The most common resistance mechanism against the beta-lactam drugs in *Pseudomonas aeruginosa* and many other nonfermenting, Gram-negative bacteria, like many members of the *Enterobacteriaceae*, is the production of AmpC beta-lactamases. AmpC beta-lactamases are responsible for inactivating a broad spectrum of beta-lactam drugs, such as penicillins, cephalosporins, and monobactams (17, 18). The most relevant mechanism for expanding the resistance phenomenon to antipseudomonal penicillins is the occurrence of mutations that lead to hyperproduction of the chromosomal cephalosporinase AmpC. Inherent to all *A. baumannii* strains are chromosomally encoded AmpC cephalosporinases, also known as Acinetobacter-derived cephalosporinases (ADCs). Unlike the AmpC enzymes found in other Gram-negative organisms, inducible AmpC expression does not occur in *A. baumannii* (5-7). The key determinant regulating the overexpression of this enzyme in *A. baumannii* is the presence of an upstream IS element known as IS*Aba*1. The presence of this element highly correlates with increased AmpC gene expression and resistance to extended-spectrum cephalosporins (15). ISAbal is 1180 bp long, with terminal inverted repeats of 16 bp (Gene bank accession no. AY758396), and belongs to the IS4 family, according to the IS nomenclature proposed by Chandler and Mahillon. Heritier et al. (19) found that the inverted left repeat of ISAbal was located 9 bp upstream of the start codon of the ampC gene. ISAbal provides a new promoter sequence that replaces the original promoter sequence that drives low level expression of AmpC in the absence of ISAbal. The insertion event also results in a nucleotide change inside the ribosome binding site sequence of the ampC gene; however, Heretier et al. were able to demonstrate that this change did not influence expression of the ampC gene, and that high level expression was associated only with the presence of the ISAbal promoter sequence. Numerous copies of ISAbal occur in *Acinetobacter* spp., but to date, it has not been found in other organisms such as the *Enterobacteriaceae* or *Pseudomonas aeruginosa* (15). ISAbal, or similar elements, has also been associated with the expression of other antibiotic resistance genes in *A. baumannii*, and is also closely related (three nucleotide changes) to the partial sequence found upstream of the pbaBAC sequence of *Acinetobacter* strain RA3849, which encodes an acetoacetyl coenzyme A reductase (20). Accordingly, our experimental data suggest that IS*Aba1* (insertion sequence element) provides a putative promoter that enhances high-level expression of the bla_{ampC} beta -lactamase gene. Furthermore, the PCR results were in accordance with the ceftazidime MIC; as previously mentioned, IS*Aba1* was absent in ceftazidime-susceptible *A. baumannii* isolates (8, 21). The cephalosporins have been accepted worldwide for the treatment of bacterial infections due to their safety and pharmacokinetic features (21-23). Despite the advent of new drugs against emergent bacterial resistance, cefepime and cefpirome, fourth-generation cephalosporins, were found to be slightly more potent than the third-generation cephalosporins tested against *Acinetobacter* spp. Cefepime appears to be stable in response to the bla_{ampC} beta -lactamase gene (22) but a high distribution of resistance to cefepime (86%) has been found in *A. baumannii* isolates; this may due to the involvement of other resistance mechanisms (other beta-lactamases, efflux, altered permeability) (17, 23). Similar to our findings, the presence

of an insertion sequence upstream of AmpC in *A. baumannii* clinical isolates from France resulted in high-level cephalosporin resistance (24). A related study, also in Iran, described resistance to cephalosporins and IS*Aba1* in *A. baumannii* that was very similar to the findings among our *A. baumannii* isolates (12). This fact indicates that resistance to the cephalosporins is increasing alarmingly. It has been suggested that the overwhelming use of antibiotics has led to the emergence of more resistant forms of colonizing strains. Interestingly, IS*Aba1* was identified upstream of the bla_{ampC} gene of *A. baumannii* isolates expressing PER-1 and VEB-1 extended-spectrum beta-lactamases, indicating a dual mechanism for cefotaxime resistance, involving both AmpC hyper-production caused by IS*Aba1* insertion and acquisition of a broad-spectrum beta-lactamase gene. In order to investigate the origin of infection, the route of spread, and the prevalence of isolates in a bacterial population, several phenotypic and molecular typing methods have been described. REP-PCR is a simple, rapid, and low-cost method that has been used to study nosocomial outbreaks of *A. baumannii* with acceptable reproducibility and discrimination (16, 25). Molecular typing by REP-PCR revealed that genotypes B and C were the most common genotypes during the study period. Clones may emerge at different locations through the independent selection of genetically related strains circulating in the community or environment as a result of antibiotic use in hospitals (26, 27). Otherwise, similar isolates may appear at different locations simply through direct spread from one hospital to another. Results of this investigation suggest that regional spread of the organism was mediated by patient transfers within regional healthcare networks. According to the results of the current research, a high percentage of *A. baumannii* isolates carrying IS*Aba*1, which is located upstream of the ampC gene, result in high-level cephalosporin particularly cefotaxime resistance.

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Footnotes

Authors Contribution: Mohammad Ahangarzadeh Rezaee and Gholamreza Zarrini were responsible for the study's concept and design, critical revision of the manuscript for important intellectual content, and study supervision. Delsuz Rezaee was responsible for drafting the manuscript, acquisition of data, and statistical analysis.

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