

Efflux Pump AdeABC Assessment in *Acinetobacter baumannii* Strains Isolated in a Teaching Hospital

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Abstract

Over the past twenty years the worldwide clinically impact of *Acinetobacter baumannii* (*A. baumannii*) demonstrated its etiopathogenetic relevance. During a previously retrospective study in a teaching hospital, between January 2011 and February 2015, we observed increasingly infections caused by *A. baumannii* associated with antibiotic multi-resistance. Tigecycline, the first member of the glycolcycline class, is an effective option for the treatment of such infections even if, due to its increased clinical use, tigecycline resistant isolates have recently emerged. In *A. baumannii* several mechanisms are associated with a tigecycline decrease susceptibility, among these, expression efflux pump AdeABC and the presence of insertion sequence (IS) in the *adeRS* operon. About that, we decided to analyze *adeB* and *adeS* genes in 24 MDR *A. baumannii* clinical isolates, selected on the different tigecycline phenotype. The study of *adeB* and *adeS* genes was performed by an *in-house* polymerase chain reaction (PCR) and by Sanger sequencing method. According to literature *adeB* and *adeS* genes were detected in all MDR *A. baumannii* isolates tested. Therefore our attention has focused on two resistant tigecycline clinical strains (ACI 2313 and ACI 1213), with a MIC value >8. In particular the ACI 2313 strains, showed the presence of an IS in the *adeS* gene. Then, *adeS* sequence analysis identified ISAb1 insertion. Moreover, *adeB* gene expression was evaluated by an *in-house* SYBR Green I-based real-time RT-PCR. We found an over expression of *adeB* gene in ACI 2313 strain, according to IS presence on *adeS* gene, while the lack of *adeB* overexpression in ACI 1213, still resistant to tigecycline, could be due to different resistance mechanisms.

Keywords: Tigecycline; *A. baumannii* isolates; Glycolcycline; Antibiotic resistance

Introduction

Acinetobacter baumannii (*A. baumannii*) is an opportunistic pathogen that commonly causes nosocomial infections, as pneumonia, bloodstream and urinary tract infections, particularly in the intensive care unit [1]. Multi drug resistant (MDR) *A. baumannii* isolates have been reported worldwide and their increasing prevalence has led to limited therapeutic choice [2].

Tigecycline, the first member of the glycolcycline class of antibacterial agents, remain effective option for the treatment of these infections. However, due to its increased clinical use, tigecycline resistance is recently emerging [3].

Several studies have indicated that tigecycline resistance of *A. baumannii* is associated with the over expression of AdeABC efflux system [4,5]. A two component system containing *adeS* and AdeR, a sensor kinase and a response regulator respectively, are responsible for modulating AdeABC efflux pump [6]. Moreover, nucleotide/amino acid variations as well as the presence of insertion sequences (IS), such as ISAb1, in the *adeRS* operon have been related to the over expression of the AdeABC efflux pump, decreasing *A. baumannii* susceptibility to tigecycline [7]. However, the exact mechanisms of resistance and the relationship between the level of expression of efflux pumps and the minimal inhibitory concentration (MIC, mg/liter) of tigecycline have not yet been clearly elucidated. Also, whether clinical isolates with resistance to tigecycline, originating from the same

geographic locations, possess similar mechanisms of resistance is still unclear.

During a retrospective study in a teaching hospital, between January 2011 and February 2015, we evaluated distribution and antibiotic resistance of *A. baumannii* strains isolated from patients admitted to four hospital units (medical units, surgical units, cardiac intensive care unit and the intensive care unit). *A. baumannii* isolates were collected from several sites such as blood culture, bronchial aspirate, bronchoalveolar lavage, central venous catheter, urine, and bladder catheter tip. Data collected showed an increasingly infections caused by *A. baumannii* associated with antibiotic multi-resistance (unpublished data). In particular on 83 strains, isolated in the last year, the percentages of MDR and pan drug resistant (PDR) *A. baumannii* were 75% and 13% respectively.

Objective

Since the observed high frequency of multi drug resistant *A. baumannii* in our hospital, the aim of this study was to assess efflux pump AdeABC in 24 MDR *A. baumannii* strains, selected on the different tigecycline phenotype.

Study Design

Twenty-four clinical isolates of *A. baumannii*, collected at "Mater Domini" University Hospital of Catanzaro, Southern Italy, from January 2013 to February 2015, were selected according to tigecycline phenotype ($0.5 \leq \text{MIC} \leq 8$). Isolates were identified by using VITEK 2 system (bioMérieux) and by mass spectrometry MALDI-TOF MS

(bioMérieux, France). *A. baumannii* ATCC 19606, an environmental *A. baumannii* strain, previously isolated from Mediterranean Sea water sample, and *A. haemolyticus* clinical isolate were also included in our study, as controls. For MALDI-TOF MS identification, bacterial cells from blood agar culture were processed according to the manufacturer's instructions. MALDI-TOF peaks were compared with reference spectra using SARAMIS integrated database. Antimicrobial susceptibility to tigecycline was determined by VITEK 2 system (AST-N201/AST-N203 cards) and United States Food and Drug Administration (FDA) breakpoint criteria for Enterobacteriaceae [8], to perform gene sequencing, bacterial DNA was extracted using UltraClean™ Microbial DNA-MoBio Kit. The *adeB* and *adeS* genes were amplified by an *in-house* PCR and following primers: *adeB* Forward (5'-TAT CCT GGT GCT ACA GCT AA-3') and *adeB* Reverse (5'-CTC GA GAC TAG ACA A TTG CC-3'), *adeS* Forward (5' GTA GAC TGG ATC TGG TTA GC-3') and *adeS* Reverse (5'-AGT GGA CGT TAG GTC AAG TT-3'). PCR products of *adeB* gene, from ACI 4614, ACI 2313, ACI 1213, and ATCC 19606 isolates, as well as *adeS* gene, from ACI 2313 and ATCC 19606 strains, were sequenced. Following amplicon purification, using PCR Illustra MicroSpin S-300 Colonne HR (Gelifesciences UK), Sanger sequencing was performed by ABI PRISM 3500 Genetic Analyzer (Applied Biosystems™, life technologies). Sequences were analyzed with BLASTN program of the NCBI Basic Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/>).

The ACI 4614, ACI 2313, ACI 1213, and ATCC 19606 isolates were also evaluated for *adeB* gene expression using an *in-house* developed SYBR Green I-based real-time RT-PCR. Total RNA was isolated by Trizol® Reagent (Ambion™, life technologies). The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) for cDNA synthesis was used. Real-Time PCR was run in a LightCycler Instrument (Roche Molecular Biochemicals, Indianapolis, IN), using same primers described above. Of each cDNA, housekeeping gene and target gene in triplicate were determined. The amplicon specificity was confirmed by melting curve analysis, previously established (*adeB* melting temperature 85.28°C ± 0.5°C). Expression level of housekeeping gene was used to normalize the abundance of the tested transcripts. Comparative threshold cycle (CT) was used to determine transcript fold changes present in ACI 4614, ACI 2313 and ACI 1213 compared to ATCC 19606 [9].

Results

The identification analyses of 24 strains were confirmed by two methods. At first, isolates were identified by VITEK 2 system. Since *Acinetobacter calcoaceticus-baumannii* (*ACB complex*) are phenotypically indistinguishable when evaluated by biochemical characteristics, the clinical isolates were also identified by mass spectrometry MALDI-TOF MS. Mass spectrometry MALDI-TOF MS identified *A. baumannii* specie with high reliability, founding m/z 5747/5749 range peaks [10]. The VITEK 2 system was also used to determine antibiotic susceptibilities. The MIC ≥8, related to tigecycline resistance, was found in ACI 1213 and ACI 2313 strains, while, the others isolates showed a MIC value ranging 0.5-4.

Following *in-house* PCR, in all MDR *A. baumannii* clinical isolates as well as in *A. baumannii* ATCC 19606 reference strain, *adeB* (Figure 1) and *adeS* (data not shown) were found. Conversely, as expected, both environmental *A. baumannii* strain and *A. haemolyticus* clinical isolates were lacking in these genes. The *adeB* Sanger sequencing from ACI 4614, ACI 1213, ACI 2313 and ATCC 19606 isolates was

performed. Sequencing analysis showed 99% homology with the *adeB* sequence of *A. baumannii* strains included in NCBI-BLAST. Additionally, in ACI 2313 isolate, *adeB* showed a threefold higher relative expression (Figure 2A and 2B). Moreover, we sequenced *adeS* gene from the same isolate (ACI 2313), showing a singular electrophoretic migration pattern, and ATCC 19606 strain, as control. Sequencing analysis demonstrated that ACI 2313 tigecycline resistant isolate carried ISAbal1 insertion sequence.

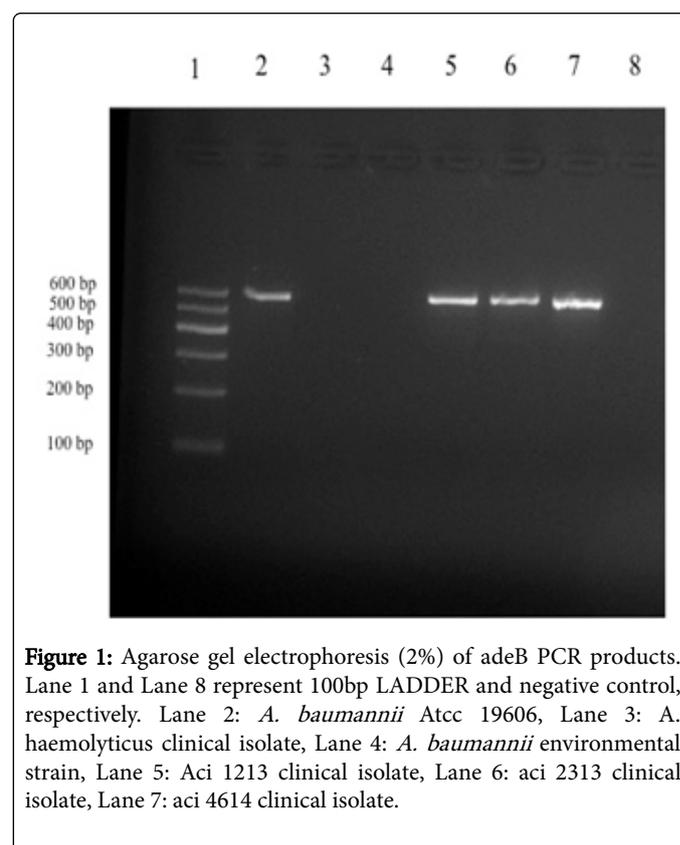


Figure 1: Agarose gel electrophoresis (2%) of *adeB* PCR products. Lane 1 and Lane 8 represent 100bp LADDER and negative control, respectively. Lane 2: *A. baumannii* Atcc 19606, Lane 3: *A. haemolyticus* clinical isolate, Lane 4: *A. baumannii* environmental strain, Lane 5: AcI 1213 clinical isolate, Lane 6: aci 2313 clinical isolate, Lane 7: aci 4614 clinical isolate.

Discussion

Different studies have reported the role of *A. baumannii* efflux pumps in resistance to clinically relevant antibiotics [11]. AdeABC efflux pump has been well characterized, it is apparently not well expressed in wild-type strains [12], and contributes significantly to acquire multi drug resistance in worldwide clinical isolates, including resistance to tigecycline increasingly reported since 2007 [13-17]. However tigecycline is one of the few remaining therapeutic options for treating infections caused by MDR *A. baumannii*. Previous report [4] suggested that decreased susceptibility to tigecycline in the complex *Acinetobacter calcoaceticus-Acinetobacter baumannii* is associated with overexpression of efflux pump AdeABC.

Recently, the overexpression of AdeABC was referred as the prevalent mechanism in tigecycline resistant *A. baumannii* clinical isolates, and a linear relationship was found between *adeB* gene expression levels and tigecycline MICs [17]. Our data showed that, even if *adeB* were detected in all MDR *A. baumannii* strains tested, differences in *adeB* gene expression have been found.

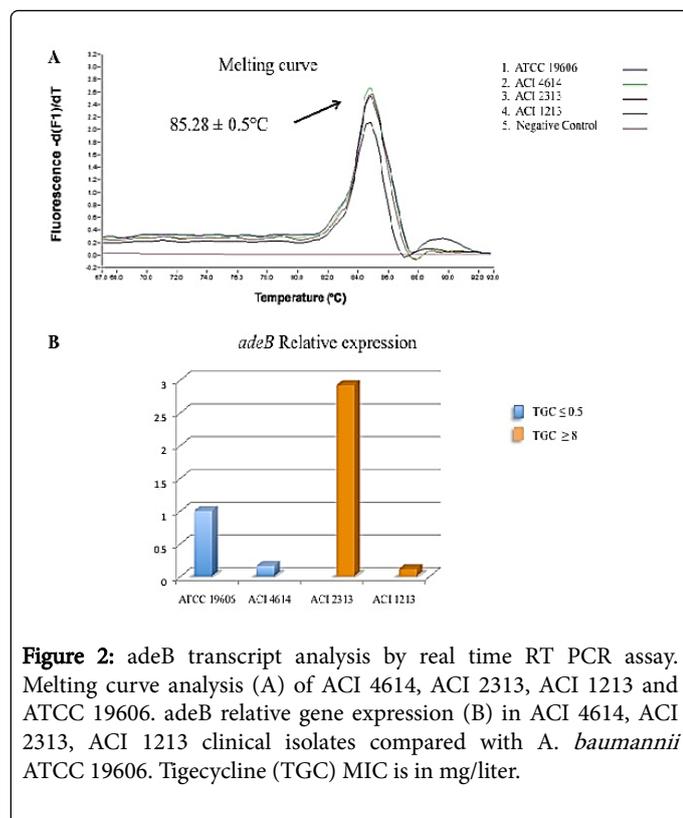


Figure 2: *adeB* transcript analysis by real time RT PCR assay. Melting curve analysis (A) of ACI 4614, ACI 2313, ACI 1213 and ATCC 19606. *adeB* relative gene expression (B) in ACI 4614, ACI 2313, ACI 1213 clinical isolates compared with *A. baumannii* ATCC 19606. Tigecycline (TGC) MIC is in mg/liter.

Indeed, in the 2 clinical isolates, sharing a MIC value >8, we determined substantial differences; in particular, in just ACI 2313 isolate, *adeB* showed a higher relative expression.

The AdeABC efflux pump is regulated by a two component system, *AdeS* sensor kinase and *AdeR* response regulator, encoded by the *adeRS* operon. It has been reported that overexpression of the AdeABC system is due to mutation in *adeRS* operon, included the presence of insertional sequence, such as ISAbal, one of the most frequent IS found in clinical isolates [6-7]. When we detected and analyzed *adeS* in our clinical isolates, ACI 2313 strain showed a distinct electrophoretic migration pattern and its partial sequencing matched to ISAbal. The analysis to cover the internal gap (around 400 bp) and to obtain the entire IS is still in progress.

In conclusion, further examination on additional tigecycline resistant *A. baumannii* clinical isolates, spreading in our area, are required. Mechanisms leading to tigecycline resistance, in particular in strains originating from the same geographic locations, will contribute to check the future prevalence of this resistance and to understand the *A. baumannii* pan drug resistance evolution.

Conflicts of interests

The authors declare that they have no competing financial interests.

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