Molecular identification of tigecycline- and colistin-resistant carbapenemase-producing *Acinetobacter baumannii* from a Greek hospital from 2011 to 2013

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An alarming increase in the resistance rates of tigecycline and colistin among carbapenemase-producing *Acinetobacter baumannii* recovered from a Greek hospital over a 3-year period (2011–2013) was investigated. The antimicrobial resistance profiles and carbapenemase gene content were determined for a collection of colistin- and/or tigecycline-resistant carbapenemase-producing *A. baumannii* isolates (*n* = 42), which were recovered consecutively during the study period. A gradual increase in the incidence of *bla*OXA-23 producers was observed from 2011 to 2013. A cluster of 21 isolates comprised tigecycline-resistant *bla*OXA-23 producers displayed a single antimicrobial resistance pattern. The emergence of two *bla*OXA-23 producers resistant to both tigecycline and colistin was documented. Furthermore, determination of the mechanisms of colistin and tigecycline resistance and molecular typing by the tri-locus sequence typing (3LST) scheme for nine isolates recovered from bloodstream infections were performed. Out of nine isolates, five tigecycline- and two colistin-resistant isolates were *bla*OXA-23 producers of 3LST ST101 corresponding to the international clone II recovered during 2012–2013. All nine isolates were positive for the presence of the adeB gene of the AdeABC efflux pump. Three colistin-resistant isolates possessed novel substitutions in PmrB, which may be implicated in colistin resistance. To the best of our knowledge, this is the first report of the acquisition of tigecycline and colistin resistance among *bla*OXA-23-producing *A. baumannii* of 3LST ST101 in Greece; thus, continuous surveillance and molecular characterization, prudent use of antibiotics and implementation of infection control measures for *A. baumannii* are urgent.

**INTRODUCTION**

Multidrug-resistant (MDR) *Acinetobacter baumannii* has emerged in recent decades as a major cause of nosocomial outbreaks and healthcare-associated infections, including bacteraemia, pneumonia, meningitis and urinary tract and wound infections. Carbapenem resistance, with rates of up to 70 % reported in some countries, can be mediated by various combined mechanisms, including the production of carbapenem-hydrolysing class D oxacillins, AmpC stable derepression, altered penicillin-binding proteins, overexpression of efflux pumps and porin loss (Bassetti *et al.*, 2008; Maragakis & Perl, 2008; Montefour *et al.*, 2008; Poulakou *et al.*, 2009; Reis *et al.*, 2003). Colistin resistance is associated with specific modification of the lipid A component of the outer membrane and is mediated by mutations in specific regions of the two-component regulation systems *pmrA/B* and *phoP/Q* (Beceiro *et al.*, 2011). Tigecycline resistance has been associated with overexpression of a variety of efflux pumps (Deng *et al.*, 2014; Hou *et al.*, 2012). The presence of the *tetX1* gene has also been associated with resistance to tigecycline (Bartha *et al.*, 2011).

<table>
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<th>Abbreviations: CLSI, Clinical and Laboratory Standards Institute; ICU, intensive care unit; 3LST, tri-locus sequence typing; MDR, multidrug resistant; ST, sequence type</th>
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Over the period 2011–2013, increases in the resistance rates of colistin and tigecycline among carbapenemase-producing \textit{A. baumannii} (from 1.3 to 18.8 \% for colistin, and from 11.3 to 27.5 \% for tigecycline) were documented at the Department of Microbiology of the General Hospital of Nea Ionia, ‘Konstantopoulou-Patission’, Athens, Greece. In order to investigate the causes of these increases, antimicrobial resistance profiles and carbapenemase gene content were determined for a collection of colistin-and/or tigecycline-resistant carbapenemase-producing \textit{A. baumannii} isolates ($n = 42$), which were recovered consecutively during the study period. Of the 42 isolates, those recovered from bloodstream infections were genotyped by the tri-locus sequence typing (3LST) scheme, and their mechanisms of resistance to colistin and tigecycline were determined.

**METHODS**

Identification, antimicrobial susceptibility testing and collection of \textit{A. baumannii} isolates. ‘Konstantopoulou-Patission’ is a general hospital in Athens (the capital city of Greece with approximately 3 million inhabitants), with a potential of 280 beds in total, including a nine-bed intensive care unit (ICU) and internal medicine, surgical, urology and other wards. Identification of the isolates to the species level and antibiotic susceptibility testing were performed using a MicroScan system (Siemens Healthcare), according to the interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI, 2013). The MICs of imipenem, meropenem, colistin and tigecycline were additionally determined using MIC Test Strips (Liofilchem S.R.L.). Following the CLSI breakpoints, isolates with MICs for imipenem and meropenem of $\geq 8 \text{ mg L}^{-1}$ and for colistin of $\geq 4 \text{ mg L}^{-1}$ were categorized as resistant. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive criteria for tigecycline (http://www.eucast.org), isolates with MICs of $\geq 4 \text{ mg L}^{-1}$ were categorized as resistant.

From January 2011 to December 2013, a total of 266 consecutive \textit{A. baumannii} isolates were defined as carbapenem resistant by antimicrobial susceptibility testing at the Department of Microbiology of the hospital. Of the 266 carbapenem-resistant \textit{A. baumannii} isolates, 42 (15.8 \%) were also defined as tigecycline resistant and/or colistin resistant and were collected for further analysis. The 42 isolates were recovered mainly from patients in the ICU (31/42 isolates; 74 \%) and from 11.3 to 27.5 \% for tigecycline) were documented during the study period. Of the 42 isolates, 28 (66 \%) were recovered from bloodstream infections, whereas the remaining seven isolates were recovered from bronchial secretions (23/42 isolates; 56 \%) and blood (9/42 isolates; 22 \%), but also from other clinical specimens (e.g. urine, sputum).

DNA extraction and detection of OXA carbapenemases and colistin and tigecycline resistance genes. DNA extraction was performed using a QIAcube system (Qiagen), according to the instructions of the manufacturer. Detection by PCR was performed for OXA type (\textit{bla}OXA-23, \textit{bla}OXA-24, \textit{bla}OXA-51 and \textit{bla}OXA-58) carbapenemase genes (Woodford \textit{et al.}, 2006), and resistance genes associated with tigecycline (\textit{adeB}, \textit{tetX1/tetX2}) and colistin (\textit{pmrB}) resistance (Bartha \textit{et al.}, 2011; Deng \textit{et al.}, 2014; Hou \textit{et al.}, 2012).

Nucleotide sequencing of the \textit{pmrB} gene. The sequences of the PCR products of \textit{pmrB} were determined on both DNA strands using an ABI3730 DNA sequencer (Applied Biosystems), according to the instructions of the manufacturer. The deduced protein sequences were aligned and compared to the WT reference sequence of \textit{A. baumannii} ATCC 17978 (GenBank accession no CP000521.1; Becerro \textit{et al.}, 2011) using MEGA4 software (Tamura \textit{et al.}, 2007).

**RESULTS AND DISCUSSION**

Antimicrobial susceptibility testing and carbapenemase gene content of colistin- and tigecycline-resistant carbapenem-resistant \textit{A. baumannii}

All colistin- and tigecycline-resistant carbapenem-resistant \textit{A. baumannii} isolates displayed MDR phenotypes, being resistant to three or more antimicrobial classes (Table 1). Of the 42 isolates, 28 were tigecycline resistant, 12 were colistin resistant and two were resistant to both tigecycline and colistin. The annual distribution of tigecycline- and colistin-resistant isolates among carbapenem-resistant \textit{A. baumannii} is shown in Fig. 1. All 42 isolates were positive for the intrinsic \textit{bla}_{OXA-51}. The \textit{bla}_{OXA-23} and \textit{bla}_{OXA-58} genes were detected in 28 and seven isolates, respectively.

It is noteworthy that seven colistin-resistant isolates carried both \textit{bla}_{OXA-23} and \textit{bla}_{OXA-58} genes (Table 1). Both \textit{bla}_{OXA-23} and \textit{bla}_{OXA-58} genes have been identified previously in an \textit{A. baumannii} isolate in Greece (Liakopoulos \textit{et al.}, 2012). A cluster of 21 isolates comprised tigecycline-resistant \textit{bla}_{OXA-23} producers and displayed a single antimicrobial resistance pattern. The annual distribution of the OXA carbapenemases among the 42 isolates is shown in Fig. 2. A gradual increase in the incidence of \textit{bla}_{OXA-23} producers was observed from 2011 to 2013.

Molecular characterization of tigecycline- and/or colistin-resistant carbapenemase-producing \textit{A. baumannii} isolates recovered from bloodstream infections

The microbiological and molecular characteristics of the isolates recovered from blood samples (nine isolates) are shown in Table 2; \textit{bla}_{OXA-58} producers (two isolates) of 3LST ST106 and ST201 were recovered during 2011, whereas the remaining seven isolates were \textit{bla}_{OXA-23} producers of 3LST ST101 recovered in 2012–2013. Of the seven \textit{bla}_{OXA-23} producers of 3LST ST101, five isolates were tigecycline resistant and two were colistin resistant. The 3LST ST101 (\textit{ompA}-\textit{csuE}-\textit{bla}_{OXA-51}-like allelic profile: 1–1–1; ST group 1) corresponds to the international clone II, which has previously been associated with divergent \textit{bla}_{OXA-23}–producing strains in several countries worldwide, including Greece (Liakopoulos \textit{et al.}, 2012; Turton \textit{et al.}, 2007; Zarrilli \textit{et al.}, 2013); thus, the acquisition of tigecycline and colistin resistance in strains of this clone is worrying for the therapeutic options and infection control policies for MDR \textit{A. baumannii}.

All nine isolates recovered from blood samples were positive for the \textit{adeB} gene (Table 2) but negative for the \textit{tetX1/tetX2} genes, as shown by PCR. In \textit{A. baumannii}, the AdeABC
Table 1. Antimicrobial resistance patterns and carbapenemase gene content of tigecycline- and/or colistin-resistant carbapenemase-producing *A. baumannii* recovered in 2011–2013

<table>
<thead>
<tr>
<th>Antimicrobial resistance pattern*</th>
<th>OXA type (no. of isolates)</th>
<th>OXA-23 + OXA-51 (n=28)</th>
<th>OXA-58 + OXA-51 (n=7)</th>
<th>OXA-23 + OXA58 + OXA-51 (n=7)</th>
<th>Total (n=42)</th>
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<tr>
<td>Tigecycline resistant (n=28)</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AMP, TIC, AMX/CLA, AMP/SUL, FEP, CAZ, ATM, IMP, MER, AK, GM, TOB, CIP, TIG</td>
<td>21 5 26</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP, TIC, AMX/CLA, AMP/SUL, FEP, CAZ, ATM, IMP, MER, AK, GM, CIP, TIG</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td>Collistin resistant (n=12)</td>
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<tr>
<td>AMP, TIC, AMX/CLA, AMP/SUL, FEP, CAZ, ATM, IMP, MER, AK, GM, TOB, CIP, COL</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP, TIC, AMX/CLA, AMP/SUL, FEP, CAZ, ATM, IMP, MER, AK, GM, CIP, COL</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td>Collistin- and tigecycline-resistant (n=2)</td>
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<tr>
<td>AMP, TIC, AMX/CLA, AMP/SUL, FEP, CAZ, ATM, IMP, MER, AK, GM, TOB, CIP, COL</td>
<td>2</td>
<td>2</td>
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*AK, amikacin; AMP, ampicillin; AMP/SUL, ampicillin/sulbactam; AMX/CLA, amoxicillin/clavulanate; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; FEP, cefepime; GM, gentamicin; IMI, imipenem; MER, meropenem; TIC, ticarcillin; TIG, tigecycline; TOB, tobramycin.

Fig. 1. Annual distribution of tigecycline- and/or colistin-resistant isolates among carbapenem-resistant *A. baumannii*. CARB-R, carbapenem resistant; TIG-R, tigecycline resistant; COL-R, colistinresistant.

![Fig. 1. Annual distribution of tigecycline- and/or colistin-resistant isolates among carbapenem-resistant A. baumannii.](image)

Fig. 2. Annual distribution of the OXA carbapenemases of tigecycline- and/or colistin-resistant carbapenemase-producing *A. baumannii* isolates.

![Fig. 2. Annual distribution of the OXA carbapenemases of tigecycline- and/or colistin-resistant carbapenemase-producing A. baumannii isolates.](image)

efflux pump is the major efflux mechanism, and increased expression of the *adeABC* genes confers resistance to β-lactams, including carbapenems, aminoglycosides and tigecycline, and decreased susceptibility to fluoroquinolones, tetracycline, chloramphenicol, erythromycin, trimethoprim and netilmicin (Deng *et al.*, 2014; Hou *et al.*, 2012).

Nucleotide sequencing of the PCR amplicons revealed the presence of four novel substitutions (Q129L, A138T, Q195H, A140S) in the *adeABC* genes.
A226V and P360Q) in the deduced amino acid sequences of PmrB in the three colistin-resistant isolates (Table 2), which may be implicated in colistin resistance. In A. baumannii, the PmrAB system is implicated in colistin resistance, and requires at least two distinct genetic events: (i) at least one amino acid change in PmrB and (ii) upregulated expression of pmrA and pmrB, although the precise genetic events that cause pmrAB upregulation have not been defined (Beceiro et al., 2011). A limitation of the present study is that pmrAB upregulation was not examined.

The rapid expansion of MDR A. baumannii and the apparent predominance of a few successful MDR lineages worldwide underline the importance of surveillance, epidemiological and evolutionary studies for this organism (Zarrilli et al., 2013). In Greece, an increase in carbapenem resistance was observed from 2005 onwards accompanied by a shift of the predominant STs among A. baumannii isolates, corresponding to the international clone II, in a Greek hospital over a 3-year period, and to the best of our knowledge, this is the first report of these isolates in Greece. Furthermore, the emergence of two blaoxa-23 producers resistant to both tigecycline and colistin is alarming and it indicates that this organism is acquiring an ever-increasing arsenal of antibiotic resistance mechanisms. Conclusively, this study demonstrates the current trends in antimicrobial resistance and the dissemination of resistant isolates to antimicrobials used for therapy in A. baumannii infections, and highlights the importance of the prudent use of antimicrobial agents, strict infection control measures and continuous surveillance of this pathogen.

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### REFERENCES


