

## High incidence of pandrug-resistant *Acinetobacter baumannii* isolates collected from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial

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**Objectives:** To investigate the molecular epidemiology, antimicrobial susceptibility and carbapenem resistance determinants of *Acinetobacter baumannii* isolates from respiratory tract samples of patients diagnosed with ventilator-associated pneumonia (VAP) who were enrolled in the MagicBullet clinical trial.

**Methods:** *A. baumannii* isolates were prospectively cultured from respiratory tract samples from 65 patients from 15 hospitals in Greece, Italy and Spain. Susceptibility testing was performed by broth microdilution. Carbapenem resistance determinants were identified by PCR and sequencing. Molecular epidemiology was investigated using rep-PCR (DiversiLab) and international clones (IC) were identified using our in-house database.

**Results:** Of 65 isolates, all but two isolates (97%) were resistant to imipenem and these were always associated with an acquired carbapenemase, OXA-23 (80%), OXA-40 (4.6%), OXA-58 (1.5%) or OXA-23/58 (1.5%). Resistance to colistin was 47.7%. Twenty-two isolates were XDR, and 20 isolates were pandrug-resistant (PDR). The majority of isolates clustered with IC2 ( $n = 54$ ) with one major subtype comprising isolates from 12 hospitals in the three countries, which included 19 XDR and 16 PDR isolates.

**Conclusions:** Carbapenem resistance rates were very high in *A. baumannii* recovered from patients with VAP. Almost half of the isolates were colistin resistant, and 42 (64.6%) isolates were XDR or PDR. Rep-PCR confirmed IC2 is the predominant clonal lineage in Europe and suggests the presence of an epidemic XDR/PDR *A. baumannii* clone that has spread in Greece, Italy and Spain. These data highlight the difficulty in empirical treatment of patients with *A. baumannii* VAP in centres with a high prevalence of carbapenem-resistant *A. baumannii*.

## Introduction

Considered susceptible to major antimicrobial drug classes in the 1970s, the clinical importance of the Gram-negative, nosocomial pathogen *Acinetobacter baumannii* has increased steadily and today this organism is displaying resistance against all first-line antibiotics.<sup>1,2</sup> Owing to its intrinsic antimicrobial resistance and its ability to easily acquire new resistance determinants, MDR, XDR and even pandrug-resistant (PDR) isolates have now been

described.<sup>1-4</sup> *A. baumannii* has established a niche in healthcare environments where it is involved in a variety of serious infections, particularly in patients with severe comorbidities in the ICU setting.<sup>1,2</sup> Infections include ventilator-associated-pneumonia (VAP), bloodstream infection, as well as wound and urinary tract infection, meningitis and soft tissue infection.<sup>1,5</sup> Of these, VAP is the most frequent healthcare-associated infection.<sup>6</sup> It is associated with excess mortality, excess length of ICU and hospital stay,

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prolonged mechanical ventilation and therefore higher overall costs.<sup>6,7</sup> The predominant organisms in nosocomial pneumonia are *Staphylococcus aureus* and aerobic Gram-negative bacilli (GNB), predominantly *Pseudomonas aeruginosa*, Enterobacteriaceae and *A. baumannii*.<sup>6,8</sup> Given the high prevalence of MDR GNB, adequate empirical antimicrobial therapy for VAP is difficult to establish and inappropriate treatment is associated with a poor prognosis in these patients.<sup>9</sup> Carbapenem monotherapy or combined with an aminoglycoside or a quinolone has become a widely applied empirical therapy.<sup>10,11</sup> However, often this strategy may not be successful as many GNB occurring in ICU settings now exhibit resistance to all three antimicrobial classes.<sup>12</sup>

Owing to a lack of new antimicrobial agents, the old off-patent drug colistin has come into consideration again, showing good *in vitro* activity against carbapenem-resistant GNB (CR-GNB).<sup>13</sup> To date, the clinical efficacy of colistin has been suggested in experimental models and clinical case series but no clinical trial using colistin as empirical treatment for VAP has been carried out.<sup>14,15</sup> Therefore, in 2012 the Phase IV, randomized, open-label, non-inferiority, international clinical trial MagicBullet was initiated to assess the safety and efficacy of colistin versus meropenem in the empirical treatment of VAP.<sup>16</sup> The study was conducted over 4 years and included 32 centres in Spain, Italy and Greece, countries with a high incidence of infections caused by CR-GNB. As part of this trial it was the aim of this study to characterize the molecular epidemiology, antimicrobial susceptibility and carbapenem resistance mechanisms of *A. baumannii* isolates recovered from respiratory tract samples from patients with VAP.

## Materials and methods

### Bacterial isolates

One hundred and twenty-four *A. baumannii* isolates were collected from respiratory tract samples (bronchoalveolar lavage or bronchial aspirates) from 65 patients diagnosed with VAP from seven hospitals in Greece (70 isolates), five hospitals in Spain (28 isolates) and three hospitals in Italy (26 isolates) between May 2012 and September 2015 as part of the clinical trial MagicBullet (ClinicalTrials.gov NCT01292031). Samples were taken before initiation of any empirical treatment for VAP, and thereafter if available at 72 h, at day 8, at the end of antibiotic treatment, and at day 28 of follow-up. Species identification and susceptibility testing were performed according to local standards. *Acinetobacter* isolates were then sent to the central laboratory for further investigation. Species identification was confirmed by *gyrB* multiplex PCR as described previously.<sup>17</sup>

### Antimicrobial susceptibility testing

The following antibacterial agents were tested (respective concentration ranges are given in parentheses): amikacin (0.125–256 mg/L); ampicillin/sulbactam (0.25/0.125–256/128 mg/L); cefepime (0.125–256 mg/L); colistin (0.03–256 mg/L); cotrimoxazole (0.06–32 mg/L); doxycycline (0.03–32 mg/L); imipenem (0.125–256 mg/L); levofloxacin (0.03–64 mg/L); meropenem (0.125–256 mg/L); minocycline (0.06–128 mg/L); sulbactam (0.06–128 mg/L); tigecycline (0.03–64 mg/L); and tobramycin (0.06–128 mg/L). Antimicrobial susceptibility testing was performed by standard broth microdilution in CAMHB according to the international standard ISO 20776-1.<sup>18</sup> Microtitre plates containing dehydrated antibacterial agents were purchased from Merlin Diagnostica (Bornheim-Hersel, Germany). Plates were incubated at 35±1 °C for 18±2 h and read visually. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains. MICs were interpreted according to the species-specific clinical breakpoints

approved by EUCAST (Version 7.0, January 2017).<sup>19</sup> As no susceptibility breakpoint for tigecycline is available for *A. baumannii*, the EUCAST breakpoint for Enterobacteriaceae was used instead (susceptible ≤1 mg/L; resistant ≥4 mg/L). Classification of MDR, XDR and PDR was based on current EUCAST breakpoints for *Acinetobacter* spp., i.e. not including tigecycline using the following *ad-hoc* definition; MDR, resistant to at least one antibiotic from three classes tested; XDR, resistant to all antibiotics tested except one; PDR, resistant to all antibiotics tested.

### PCR and sequencing of *bla*<sub>OXA</sub> genes

Detection of intrinsic and acquired OXA-type carbapenemases and presence of IS elements adjacent to genes encoding OXA-51-like, OXA-23-like and OXA-58-like was performed by PCR and *bla*<sub>OXA</sub> genes were sequenced using primers as previously described.<sup>20,21</sup>

### Molecular epidemiology

The molecular epidemiology of the isolates was determined by repetitive sequence-based PCR (DiversiLab™ System; bioMérieux).<sup>17</sup> Clonal relatedness was analysed with the DiversiLab software using the Pearson correlation statistical method. Isolates showing ≥98% similarity in their rep-PCR pattern were considered identical. Using the modified Kullback–Leibler statistical method, isolates were assigned to international clones (IC) using our in-house database and isolates that clustered at >95% were considered related to the IC.<sup>17</sup>

## Results

### Bacterial isolates

Of the total 124 *Acinetobacter* isolates that were confirmed as *A. baumannii* using the *gyrB* multiplex PCR, 65 were selected based on being the first isolate per patient and analysed. Among these 65 isolates, the majority were collected before initiation of empirical antimicrobial treatment (DT0, *n* = 26) or within 72 h of treatment (DT3; *n* = 16). The remaining isolates were recovered at 8 days of treatment (DT8; *n* = 11), at the end of antibiotic treatment (DT-F; *n* = 8) and at 28 days of follow-up (DT28; *n* = 4). All following data are based on the first isolate per patient, i.e. 65 isolates.

### Antimicrobial susceptibility

Antimicrobial susceptibility results are summarized in Table 1. The vast majority of isolates (96.9%) were determined to be resistant to imipenem, with the exception of two isolates, one each from Italy and Greece. Thirty-one isolates (47.7%) were resistant to colistin and 34 isolates were susceptible, with an MIC<sub>50</sub> of 2 mg/L and MIC<sub>90</sub> of 256 mg/L. Isolates from Greece exhibited the highest rate of colistin resistance (56.8%), whereas isolates from Italy and Spain had resistance rates of 42.9% and 28.6%, respectively. Furthermore, four, two and one isolates from Greece, Italy and Spain had colistin MICs ≥256 mg/L, respectively. Regarding tigecycline, five isolates exhibited a resistant phenotype (7.7%), 23 were intermediate (35.4%) and 37 isolates (56.9%) were considered susceptible. Tigecycline MIC<sub>50</sub> and MIC<sub>90</sub> values were 1 and 2 mg/L, respectively.

Susceptibility testing against the other antibiotics revealed high overall resistance rates to the aminoglycosides and levofloxacin (Table 1). One isolate was fully susceptible to all antibiotics tested, 21 were considered MDR, 22 were XDR and 20 were PDR. There was no difference in resistance rates by country (data not shown).

**Table 1.** Susceptibility data of *A. baumannii* isolates (first isolates only, *n* = 65) collected from patients with VAP in Greece, Italy and Spain

Antimicrobial agent	MIC <sub>50</sub> <sup>a</sup>	MIC <sub>90</sub>	Range	%S <sup>b</sup>	%I	%R
Amikacin	>256	>256	1->256	6.2	7.7	86.1
Ampicillin/sulbactam	32/16	64/32	1/0.5-128/64	-	-	-
Cefepime	64	128	2->256	-	-	-
Colistin	2	256	0.25->256	52.3	-	47.7
Cotrimoxazole	32	>32	0.125->32	26.2	1.5	72.3
Doxycycline	>32	>32	0.125->32	-	-	-
Imipenem	32	64	0.25-128	1.5	1.5	96.9
Levofloxacin	16	64	0.125->64	1.5	-	98.5
Meropenem	64	64	0.25-128	3.1	9.2	87.7
Minocycline	8	16	0.125-16	-	-	-
Sulbactam	16	32	1-64	-	-	-
Tigecycline <sup>c</sup>	1	2	0.125-8	56.9	35.4	7.7
Tobramycin	>128	>128	0.125->128	21.5	-	78.5

<sup>a</sup>MIC values are given in mg/L.

<sup>b</sup>S, susceptible; I, intermediate; R, resistant; -, no EUCAST breakpoint available.

<sup>c</sup>Enterobacteriaceae breakpoints were applied for tigecycline MIC interpretation; S ≤ 1 mg/L; R ≥ 4 mg/L.

### Detection of carbapenemases

The *A. baumannii* isolates were investigated for the presence of intrinsic and acquired OXA-type carbapenemases (Table 2). Among the intrinsic OXA-51 subclass, OXA-66 was predominant, present in 54 of 65 isolates (83%) collected from all three countries. Five isolates from Greece had OXA-69, whereas three isolates from Italy carried OXA-82, which was associated with IS*Aba1* encoded upstream. Furthermore, two isolates from one hospital in Spain harboured OXA-387. A single carbapenem-susceptible isolate from Italy was found to carry OXA-391, and no further acquired OXA-type carbapenemases were detected in this isolate. Similarly, one of the OXA-69 harbouring isolates did not carry any additional OXA, and was susceptible to the carbapenems.

Among the acquired carbapenem resistance genes, OXA-23 associated with IS*Aba1* encoded upstream was found in 80% (*n* = 52) of isolates. OXA-58 associated with IS*Aba3* (*n* = 6) was found in Spain and Italy, and OXA-40 (*n* = 3) was only detected in isolates from Spain; only one isolate from Italy harboured OXA-58. Furthermore, one isolate from Athens, Greece harboured both OXA-23 and OXA-58.

### Molecular epidemiology

The majority of the isolates clustered with international clonal lineages IC1 and IC2 (Table 3). Five isolates from Greece clustered with IC1 strains from our in-house database, four of which were from one hospital in Greece and showed ≥98% similarity (cluster A; Figure 1). Eighty-three per cent of isolates (*n* = 54) exhibited a rep-PCR pattern characteristic for IC2 strains, 53 of which clustered with ≥98% similarity (cluster B) and comprised isolates from all three countries, and one isolate displayed a unique rep-PCR pattern.

**Table 2.** Percentage of isolates (*n* = 65) with the following intrinsic and acquired OXA-type carbapenemases

OXA-type	Percentage of isolates
Intrinsic carbapenemases	
OXA-66	83.1
OXA-69	7.7
OXA-82 <sup>a</sup>	4.6
OXA-387	3.1
OXA-391	1.5
Acquired carbapenemases	
OXA-23 <sup>a</sup>	80.0
OXA-40	4.6
OXA-58 <sup>b</sup>	10.8
OXA-23 <sup>a</sup> /OXA-58 <sup>b</sup>	1.5
none	3.1

<sup>a</sup>Associated with IS*Aba1*.

<sup>b</sup>Associated with IS*Aba3*.

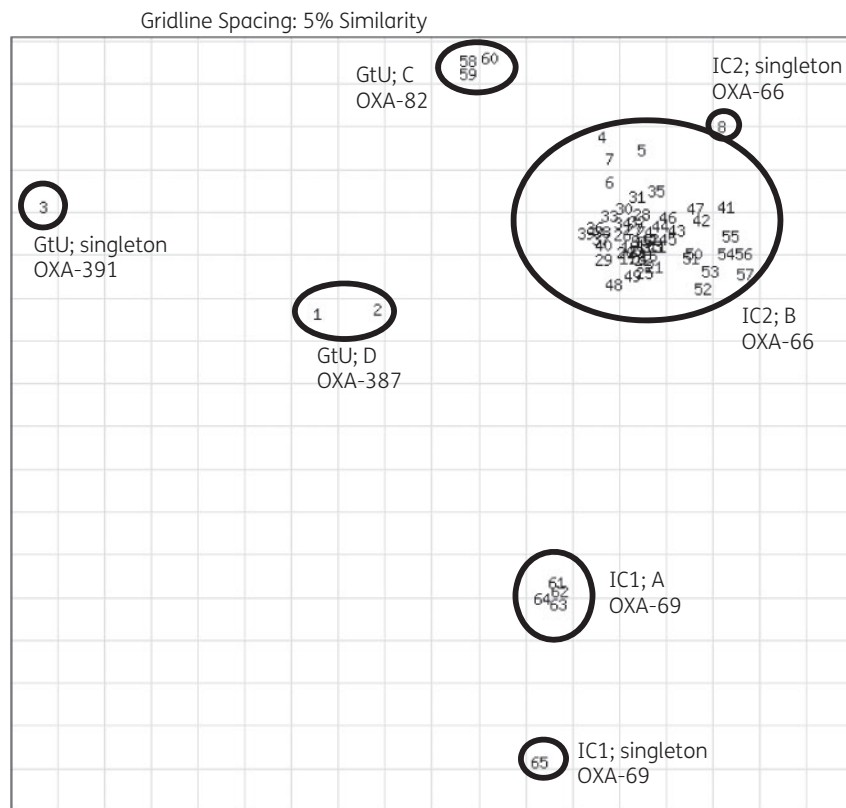
**Table 3.** Molecular typing and clustering of 65 *A. baumannii* isolates

Lineage	rep-PCR cluster	No. of isolates	Country (no. of hospitals)
IC1	A	4	Greece (1)
	singleton	1	Greece (1)
IC2	B	53	Spain (4), Italy (2), Greece (6)
	singleton	1	Spain (1)
Genotypically unique	C	3	Italy (2)
	D	2	Spain (1)
	singleton	1	Italy (1)

Sixteen of 20 PDR isolates and 19 of 22 of the XDR isolates were from cluster B. Furthermore, six isolates from Italy and Spain could not be assigned to any of the eight previously described ICs and were considered genotypically unique. Of these, three isolates from two hospitals in Italy formed cluster C (all were PDR), two isolates from one hospital in Spain represented cluster D, and one isolate from Italy had a unique rep-PCR pattern.

All IC1 isolates had the OXA-69 variant encoded on the chromosome, whereas all IC2 isolates had the OXA-66 variant. Isolates harbouring OXA-82, OXA-387 or OXA-391 each had a genotypically unique rep-PCR pattern.

Thirty patients were treated with colistin, of which 18 had strains resistant to colistin, compared with 13 of 35 colistin-resistant strains in patients who were treated with meropenem. Typing showed that there was sometimes more than one colistin-resistant strain within a hospital, suggesting that resistance was developing independently. Furthermore, we observed in some hospitals that colistin-resistant clones persisted over time and were recovered from patients irrespective of the patient receiving meropenem or colistin. We also observed colistin-resistant isolates first appearing in meropenem-treated patients and the strain spreading to other patients.



**Figure 1.** DiversiLab scatterplot of 65 *A. baumannii* isolates with their respective DiversiLab clustering and intrinsic  $bla_{OXA}$ . GtU, genotypically unique; A–D, clusters, based on rep-PCR typing.

## Discussion

The prevalence of MDR, XDR and in particular PDR *A. baumannii* is increasing worldwide, limiting the treatment options for infected patients. As part of the MagicBullet clinical trial, this study characterized *A. baumannii* isolates recovered from respiratory tract samples from patients with VAP to gain insights into the current clinical epidemiology of *A. baumannii* isolates from southern European countries (Greece, Italy and Spain), which are notorious for a high incidence of MDR and XDR *A. baumannii*.

In our *A. baumannii* isolates, we found a clear predominance of IC2, which is widely distributed around the world.<sup>17</sup> Furthermore, in longitudinal studies carried out over a 10 year period in Greece and Spain, a shift from other lineages, in particular from IC1 to IC2 has been observed,<sup>22,23</sup> and only five isolates from two hospitals in Greece belonged to IC1 in our study.

IC1 and IC2 isolates encoded the intrinsic OXA-type carbapenemase genes  $bla_{OXA-69}$  or  $bla_{OXA-66}$ , respectively, as previously described.<sup>24</sup> It has been suggested that the emergence of these two lineages may play a role in the spread of carbapenem resistance, due to the acquired carbapenemases they harbour,<sup>25</sup> and we found with only one exception, all isolates assigned to IC1 or IC2 were resistant to carbapenems. The resistant phenotype was always associated with acquired carbapenemases, with OXA-23 being the most prevalent. OXA-23 was first described in Italy, Greece and Spain in 2009, 2011 and 2013, respectively, and has now almost completely replaced OXA-58 in these countries, a phenomenon that has also been described in other countries.<sup>25–28</sup>

Overall, carbapenem resistance was almost universal in the isolates from our study confirming or even exceeding the resistance rates reported previously in the three countries.<sup>29–31</sup> This spread and high incidence of carbapenem-resistant isolates has been attributed to the extensive use of carbapenems for the treatment of infections caused by MDR Gram-negative bacteria, including *A. baumannii*. In addition, carbapenem-resistant isolates within the present study also exhibited high resistance rates to other  $\beta$ -lactams, aminoglycosides, fluoroquinolones, tetracyclines (with the exception of tigecycline) and cotrimoxazole as previously reported.<sup>29</sup> For these reasons, tigecycline and, in particular, colistin, were considered last resort antimicrobials for the treatment of patients infected with CR *A. baumannii*.<sup>29,30</sup> However, over the course of this study, colistin resistance rates were already initially high and increased from 32% in 2012–13, to 62% in 2014–15, respectively. Colistin resistance is still relatively rare in *A. baumannii* although this might be due to the difficulties in susceptibility testing. A recent study suggests that colistin resistance is greatly underestimated using commercial susceptibility testing methods.<sup>32</sup> We do not have the original laboratory-generated colistin susceptibility data for all the strains in this study. Where we have these data, of 38 samples, 5 recorded higher MICs in the local laboratories compared with broth microdilution, and these 5 were shown to be falsely reported as resistant. Thirty isolates recorded lower MICs in the local laboratories compared with broth microdilution and of these, 18 were shown to be falsely reported as susceptible. Four isolates recorded the same MICs as reported by the local



laboratory and broth microdilution. These data reinforce the idea that colistin resistance is probably underreported. We found that the highest incidence of colistin resistance was from Greece, and in a recent study 27% of *A. baumannii* from Greece were colistin resistant, while in Germany, a PDR strain was isolated from a patient who had been hospitalized in Greece.<sup>4,33</sup>

Although there is a published definition for PDR,<sup>34</sup> we did not apply this to the present study because many of the antibiotics listed have no EUCAST breakpoint values set for *Acinetobacter* spp. The XDR and PDR isolates we found in all three countries had very similar rep-PCR patterns suggesting clonal relatedness. Previously we have witnessed the importation of a PDR strain from Greece into Germany, which highlights the danger these organisms pose not only in their country of origin, but globally.<sup>4</sup>

In conclusion, among *A. baumannii* isolates recovered from patients with VAP in southern Europe, carbapenem resistance was almost universal. Alarming, 32% ( $n = 21$ ) of isolates were MDR, 34% ( $n = 22$ ) were XDR and 31% ( $n = 20$ ) PDR, highlighting the difficulty in selecting antimicrobial treatment regimens for patients with *A. baumannii* VAP. Rep-PCR revealed IC2 as the predominant lineage with 82% of isolates grouping in one large cluster, which included the majority of the PDR isolates, suggesting the presence of an epidemic PDR *A. baumannii* clone that has spread within Greece, Italy and Spain.

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## Other members of the MagicBullet Study Group Working Group WP4

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