Short Communication

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Repetitive-DNA-element PCR fingerprinting and antibiotic resistance of pan-European multi-resistant Acinetobacter baumannii clone III strains

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In the present study, it was shown that repetitive-DNA-element PCR fingerprinting using the (GTG)5 primer [(GTG)5-PCR] is a rapid and reliable tool to genotypically differentiate members of the recently described pan-European multi-resistant Acinetobacter baumannii (MAB) clone III from the known MAB clones I and II. The identification of four new representatives of the MAB clone III dating from 1991 to 1993 by (GTG)5-PCR indicates that this clone has persisted in European hospitals since the beginning of the 1990s. Tetracycline (TET) resistance was found to be common among clone III strains, including one strain that also displayed resistance to minocycline. The TET-resistance phenotype in this MAB clone appeared to be strongly associated with the presence of the efflux-type gene tet(A), but the fact that some members lack this gene or have acquired an additional tet gene [i.e. tet(M)] suggests that the tet gene carriage in the pan-European clone III population may have diversified in time and space. In contrast, all clone III strains shared the previously described aminoglycoside resistotype encoded by the aminoglycoside-modifying genes aphA6 and the class 1 integron-associated aadB, which may point to the fact that these genes probably are more stably inherited in MAB clone III compared to tet genes.

Introduction

Bacteria of the species Acinetobacter baumannii are important nosocomial pathogens (Bergogne-Bérézin & Towner, 1996). Many A. baumannii infections are outbreaks of epidemiologically successful strains that have acquired resistance to multiple antibiotics and are able to persist for a prolonged period in hospital environments (Canton et al., 2003). In recent literature, several examples of endemic cases (Kuo et al., 2004; Lopez-Otsoa et al., 2002; Quale et al., 2003) and epidemic outbreaks, such as those associated with the pan-European clones (Dijkshoorn et al., 1996; Nemec et al., 2004a) of multi-resistant A. baumannii (MAB), have been described. For the purpose of long-term intervention strategies and for the recognition of newly emerging resistance types, it is vitally important that MAB can be discriminated at the individual strain and/or clonal level. Ribotyping, amplified fragment length polymorphism (AFLP) analysis, PFGE of macro-restriction fragments and repetitive DNA element (rep)-PCR are, amongst others, by far the most frequently used genotypic methods for MAB typing (Bou et al., 2000; Brisse et al., 2000; Dijkshoorn et al., 1996). Whereas most of the aforementioned techniques require specialized equipment and/or are relatively expensive and time-consuming, rep-PCR fingerprinting is a simple and low-cost method that allows grouping of MAB strains with various degrees of genotypic relatedness.

We recently applied rep-PCR using the (GTG)5 primer [(GTG)5-PCR] to assess the genotypic relatedness among a set of tetracycline (TET)-resistant MAB strains (Huys et al., 2005). This study indicated that members of pan-European
clones I and II (Dijkshoorn et al., 1996) grouped into distinct (GTG)5-PCR clusters and that most but not all members of clones I and II contained one specific TET-resistance gene (tet gene), i.e. tet(A) or tet(B), respectively. In contrast, Nemec et al. (2004b) reported that MAB clones I and II displayed a remarkable intraclonal heterogeneity of aminoglycoside (AG)-resistance genes and structural types of class 1 integrons, whereas the recently described pan-European clone III (van Dessel et al., 2004) appeared to be homogeneous for these properties. In this context, the current study set out to further explore the discriminatory potential of the (GTG)5-PCR method to differentiate MAB clone III from clones I and II and to investigate the possible association of previously and newly described clone III members with specific TET- and AG-resistance genes.

**Methods**

The 12 *A. baumannii* strains studied included eight representatives of PFGE types 10 and 11 of pan-European clone III (van Dessel et al., 2004) and four strains isolated from four different patients of the University Hospital of Ghent, Belgium (Table 1) that were previously grouped by (GTG)5-PCR fingerprinting in a cluster clearly distinct from pan-European clones I and II (Huys et al., 2005). The four Belgian strains were resistant to TET and to several AGs. Two newly described clone III strains (Table 1) were deposited in the public BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium (http://www.belspo.be/bccm/db/bacteria_search.htm) as LMG 22452 and LMG 22863. (GTG)5-PCR fingerprinting was performed as described previously (Gevers et al., 2001). AFLP fingerprinting was performed according to Nemec et al. (2001) and PFGE analysis was carried out as described by van Dessel et al. (2004) except that 30 U Apal was used and that electrophoresis was done for 19 h with pulse times ramping from 5 to 20 s. DNA fingerprints were analysed using the BioNumerics v 3.5 software package (Applied Maths). New (GTG)5-PCR fingerprints were compared using a BioNumerics database consisting of fingerprints of mainly clinical MAB strains previously assigned to pan-European clones I and II but also including other genotypically related and unrelated strains (Huys et al., 2005; Fig. 1). The similarity among (GTG)5-PCR profiles was calculated using the Pearson correlation coefficient and visualized in a mean linkage (UPGMA) dendrogram.

The MICs of TET (range 4–512 μg ml⁻¹) and minocycline (MIN, range 1–128 μg ml⁻¹) were determined according to a microbroth dilution method using MH II broth (Becton Dickinson) following the method established by the NCCLS (2003). Susceptibility to AGs was determined by the disc diffusion method according to the NCCLS (2000) recommendations using the following discs (Oxoid): kanamycin (30 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg) and netilimicin (30 μg).

PCR-based detection of the TET-resistance genes tet(A), tet(B), tet(C), tet(D), tet(E), tet(H) and tet(M) was determined using previously described primer sets and positive control strains (Huys et al., 2005). The presence of the AG-resistance genes aphA1, aphA6, aacC1, aacC2, aacA4, aadB and aadA1, and their association with class 1 integrons through detection of the integrase gene intI1 was assessed as previously reported (Nemec et al., 2004b). The presence of the multidrug efflux gene adeD previously detected in *A. baumannii* was verified using the primer pair O3 and O4, which targets a 979 bp fragment internal to this gene (Magnet et al., 2001).

### Table 1. Phenotypic and genotypic characterization of TET resistance in pan-European *A. baumannii* clone III strains

<table>
<thead>
<tr>
<th>Original strain no.</th>
<th>Clinical source</th>
<th>Geographical origin</th>
<th>Year of isolation</th>
<th>Reference</th>
<th>MIC (μg ml⁻¹)</th>
<th>tet gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TET</td>
<td>MIN</td>
</tr>
<tr>
<td>UZG S91 00973</td>
<td>Sputum</td>
<td>Ghent, B</td>
<td>1991</td>
<td>Huys et al. (2005)</td>
<td>256</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>UZG S91 04600</td>
<td>Sputum</td>
<td>Ghent, B</td>
<td>1991</td>
<td>Huys et al. (2005)</td>
<td>256</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>(=LMG 22452)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UZG F93 03448</td>
<td>Intensive care unit patient</td>
<td>Ghent, B</td>
<td>1993</td>
<td>Huys et al. (2005)</td>
<td>256</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>(=LMG 22863)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12A133</td>
<td>Blood</td>
<td>Utrecht, NL</td>
<td>1997</td>
<td>van Dessel et al. (2004)</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>06A201</td>
<td>Blood</td>
<td>Lille, F</td>
<td>1997</td>
<td>van Dessel et al. (2004)</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>16D083</td>
<td>Wound</td>
<td>Sevilla, S</td>
<td>1997</td>
<td>van Dessel et al. (2004)</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>04C048</td>
<td>Respiratory tract infection</td>
<td>Paris, F</td>
<td>1997</td>
<td>van Dessel et al. (2004)</td>
<td>256</td>
<td>2</td>
</tr>
<tr>
<td>18A155</td>
<td>Blood</td>
<td>Barcelona, S</td>
<td>1997</td>
<td>van Dessel et al. (2004)</td>
<td>256</td>
<td>4</td>
</tr>
<tr>
<td>16D025</td>
<td>Wound</td>
<td>Sevilla, S</td>
<td>1998</td>
<td>van Dessel et al. (2004)</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>10C070</td>
<td>Respiratory tract infection</td>
<td>Genoa, I</td>
<td>1998</td>
<td>van Dessel et al. (2004)</td>
<td>256</td>
<td>1</td>
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<tr>
<td>17C085</td>
<td>Respiratory tract infection</td>
<td>Madrid, S</td>
<td>1998</td>
<td>van Dessel et al. (2004)</td>
<td>256</td>
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</table>
Results and Discussion

Identification of MAB clone III strains by (GTG)$_5$-PCR

The pan-European clones I and II were first found in several north-west European countries (Dijkshoorn et al., 1996) and, more recently, also appeared to be widespread in the Czech Republic (Nemec et al., 2004a). A third lineage, clone III, was recently identified among clinical isolates from four European countries (van Dessel et al., 2004). For clinical and epidemiological purposes, it is important that members of these and other emerging MAB clones can be rapidly recognized, preferably by using simple and standardized methods.

In the present study, eight MAB clone III strains originating from the SENTRY surveillance program (Brisse et al., 2000) were selected from different European hospitals and/or from different time periods to investigate their genotypic grouping by (GTG)$_5$-PCR fingerprinting (Table 1). Comparison of the newly generated (GTG)$_5$-PCR profiles with database profiles revealed that the pan-European clone III strains constituted a distinct cluster that was well separated from the clusters representing clones I and II and from ungrouped MAB strains (Fig. 1). This grouping remained stable over two subsequent but independent (GTG)$_5$-PCR runs, and an inter-gel reproducibility of 93.2% was obtained when including clone II strain BM4454 as a reference during each run.

When the database fingerprints of other European MAB hospital isolates unrelated to clones I and II (Huys et al., 2005) were added to this cluster analysis, it was found that four additional strains from Belgium clearly grouped with clone III, at a delineation level of 75-1% (Fig. 1). To further verify their genotypic relatedness to clone III, the four strains were also subjected to AFLP (van Dessel et al., 2004). Cluster analysis of digitized AFLP fingerprints indeed confirmed the classification of the four Belgian strains as members of clone III, with a Pearson product-moment correlation coefficient of 88% (data not shown), which is well above the 80% cut-off level used by van Dessel et al. (2004) for the delineation of MAB clones. Collectively, the DNA fingerprinting data thus indicate that the prevalence of MAB clone III strains across Europe is not limited to France, the Netherlands, Italy and Spain, but also includes Belgium (Table 1). Furthermore, the fact that the four Belgian strains were collected in the period 1991–1993 whereas all strains in the original description of clone III date from 1997–1998 (van Dessel et al., 2004) suggests that this clone has persisted in European hospitals for more than 8 years.

Originally, pan-European MAB clone III was delineated by molecular typing methods that require expensive and specialized equipment, such as the RiboPrinter (for ribotyping) or an automated DNA sequencer (for AFLP), or that are relatively time-consuming, such as PFGE (Brisse et al., 2000; van Dessel et al., 2004). Our results reinforce that (GTG)$_5$-PCR as a simple, rapid and low-cost fingerprinting method is equally suited for the delineation of the major pan-European MAB clones. This study also shows that storage of (GTG)$_5$-PCR fingerprints, which have been generated and processed under standardized conditions, in a BioNumerics database allows early recognition of new members of emerging MAB clones such as clone III. Although it has been reported that the discriminatory power of rep-PCR may be comparable to that of PFGE (Bou et al., 2000), the latter method and AFLP probably are more powerful to unravel genomic variations at a subclonal level within MAB clones.

The (GTG)$_5$-PCR band patterns of three out of the four newly identified clone III strains were not significantly different in composition from the other strains of this clone (Fig. 1). However, relatively low linkage levels were obtained in overall clustering analysis, which were mainly caused by variations in relative band intensities and background signals. Visual comparison (Fig. 2a) indicated the presence of two (GTG)$_5$-PCR types among the Belgian clone III strains, i.e. G1 (represented by three strains) and G2 (strain UZG S91 02796 = LMG 22452). In comparison, macro-restriction analysis with ApaI showed that the four Belgian strains were indistinguishable and all belonged to the same PFGE type P1 (Fig. 2b), whereas in AFLP analysis the four strains were found to represent three different genotypes on the basis of three or more band differences (A1, A2 and A3; Fig. 2c). These data again illustrate that estimation of genetic variation within major MAB clones requires the combined use of multiple fingerprinting methods displaying different levels of intra-clonal resolution. In this regard, the continuous accumulation of polyphasic DNA fingerprinting data into well-structured databases will prove to be extremely useful as a reference framework for future evaluations of sequence-based typing methods in MAB epidemiology and ecology.

Characterization of antibiotic resistance in MAB clone III

Several studies have indicated that members of the same MAB clone with highly similar if not indistinguishable genotypes can show considerable variation in their respective antibiotic-resistance properties (Dijkshoorn et al., 1996; Nemec et al., 2004b). Previously, van Dessel et al. (2004) reported that clone III strains displayed resistance to several AGs and quinolones. Like all previously investigated members of pan-European clones I and II (Huys et al., 2005), the 12 strains of clone III included in the present study contained the adeB gene. This aspecific drug efflux gene was first described in A. baumannii strain BM4454, in which it conferred resistance to several AGs and quinolones. All eight previously investigated members of pan-European clones I and II (Huys et al., 2005) also showed resistance to several AGs (Magnet et al., 2001), but seems to be widespread among European MAB clones. As opposed to pan-European MAB clones I and II, Nemec et al. (2004b) found that six members of clone III from Spain, France and the Netherlands all shared one specific AG resistotype (i.e. resistance to kanamycin, gentamicin, tobramycin and amikacin), which in all cases was encoded by the AG-modifying genes aphA6 and aadB. The latter gene was inserted as a gene cassette in a 0.75 kb variable region of a
class 1 integron (Nemec et al., 2004b). Likewise, the previously untested clone III strains 10C070 and 16D083, and the four Belgian strains also displayed the same AG resistotype and, of all seven AG-modifying genes analysed, only harboured *aphA6* and class 1 integron-associated *aadB* (data not shown). This observation may indicate that the latter MAB clone has undergone little or no diversification of AG-resistance traits in the period 1991–1998.

In contrast to AG resistance, the TET-resistance properties of pan-European MAB clone III appear to be less uniform. Based on a MIC breakpoint of $\geq 16 \mu g ml^{-1}$ (NCCLS, 2003), 11 out of the 12 strains were classified as TET resistant, with most strains displaying MICs in the range of 128–256 $\mu g ml^{-1}$ (Table 1). Compared to previously reported MIC data (Huys et al., 2005), phenotypic resistance levels of clone III to TET generally resembled those of MAB clone I (range of 32 to $\geq 512 \mu g ml^{-1}$) but were lower than those of MAB clone II ($\geq 512 \mu g ml^{-1}$). On the other hand, all strains were susceptible (MIC $\leq 4 \mu g ml^{-1}$) to the second-generation TET compound MIN except for the intermediate-resistant strain LMG 22452, which showed a MIC of 8 $\mu g ml^{-1}$. On the basis of these *in vitro* susceptibility data, MIN could be considered as a valuable agent to treat MAB clone III infections.

By PCR detection of *tet* genes (Table 1), it was found that 10 out of 12 MAB clone III strains contained the efflux type gene *tetA* whereas strain LMG 22452 harboured, in addition to *tetA*, the ribosomal protection type gene *tetM*, which

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**Fig. 1.** Dendrogram showing clustering of digitally inverted (GTG)$_4$-PCR fingerprints of MAB clone III strains in relation to database profiles of clones I and II (abridged clusters) and ungrouped *A. baumannii* strains. The dendrogram was obtained with the unweighted paired group method using arithmetic averages (UPGMA). Newly identified members of clone III are indicated in bold. DGK, Faculteit Diergeneeskunde, Ghent University, Ghent, Belgium; HPA, Health Protection Agency, London, UK; NIPH, Collection of A. Nemec, National Institute of Public Health, Prague, Czech Republic; RUH, Collection of L. Dijkshoorn, Leiden University Medical Center, Leiden, the Netherlands; UZG, Universitair Ziekenhuis Ghent, Ghent, Belgium.
confers resistance to MIN. Interestingly, the latter strain could also be differentiated from the three other Belgian strains on the basis of (GTG)₅-PCR and AFLP fingerprinting (Fig. 2). As expected, the TET-susceptible strain 16D025 did not possess any of the studied tet genes. Similar to most members of clone I (Huys et al., 2005), TET resistance in clone III strains thus appears to be strongly associated with the presence of tet(A). However, the fact that some members lack this gene (strain 16D025 from Spain and strain 12A133 from the Netherlands) or have acquired an additional tet gene (strain LMG 22452 from Belgium) suggests that the tet gene carriage of the pan-European clone III population has diversified in time and space. Likewise, van Dessel et al. (2004) pointed out that susceptibilities to piperacillin/tazobactam in clone III were highly variable and may reflect differences in antibiotic pressure possibly leading to successful mutations or to the acquisition of mobile resistance elements.

In conclusion, this study supports previous findings showing that (GTG)₅-PCR is a cost-effective tool for the rapid recognition of new members of major pan-European MAB clones such as clone III. The finding that the oldest clone III strains described so far date from 1991 indicates that this clone has spread among European hospitals for several years, as is also the case for MAB clones I and II. Furthermore, our data suggest that the degree of diversification in antibiotic-resistance traits among clone III strains may be higher for tet genes compared to AG-modifying genes, but more strains need to be investigated to support this conclusion. This finding may possibly be associated with the high frequency and efficiency by which tet gene carriers can successfully disseminate throughout MAB populations.

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References


