Emergence of OXA-carbapenemase- and 16S rRNA methylase-producing international clones of Acinetobacter baumannii in Norway

Nabil Karah,1,2 Bjørg Haldorsen,1 Nils O. Hermansen,3 Yngvar Tveten,4 Eivind Ragnhildstveit,5 Dag H. Skutlaberg,6 Ståle Tofteland,7 Arnfinn Sundsfjord1,2 and Ørjan Samuelsen1

1Reference Centre for Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway
2Research Group for Host–Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway
3Department of Microbiology, Oslo University Hospital Ullevål, Oslo, Norway
4Department of Microbiology, Vestfold Hospital, Tønsberg, Norway
5Department of Microbiology, Østfold Hospital, Fredrikstad, Norway
6Department of Microbiology, Haukeland University Hospital, Bergen, Norway
7Department of Microbiology, Sørlandet Hospital, Kristiansand, Norway

This study was designed to investigate the molecular epidemiology and antibiotic-resistance characteristics of 11 carbapenem-resistant clinical isolates of Acinetobacter baumannii obtained in Norway between 2004 and 2009. Interestingly, all the isolates were linked with recent hospitalization outside Norway. The epidemiological status was investigated by multilocus sequence typing (MLST), multiplex PCR assays for major international clones, typing of blaOXA-51-like variants and PFGE. The genotypic-resistance characteristics, including the occurrence of OXA-carbapenemase-encoding and 16S rRNA methylase-encoding genes and class 1 integrons, were investigated by PCR assays and sequencing. Seven isolates were found to harbour blaOXA-66 and belong to MLST clonal complexes (CCs) CC2P (Pasteur Institute scheme) and CC92B (Bartual scheme), and international clone II. One isolate harboured blaOXA-69, and belonged to CC1P, CC109B and international clone I. Two isolates belonged to sequence group 9, probably a subgroup of international clone I, and one isolate belonged to sequence group 4, a proposed novel international clone. All isolates contained an acquired OXA-carbapenemase-encoding gene: blaOXA-23-like (n=9), blaOXA-24-like (n=1) and blaOXA-58-like (n=1). Four isolates with high-level aminoglycoside-resistance contained the 16S rRNA methylase-encoding armA gene. Class 1 integrons with six different variable regions were detected. Sequence analysis of gene cassettes identified four aminoglycoside (aacA4, aac(6’)-Im,aadA1 and aacC1), two chloramphenicol (catB8 and cm1A5), one β-lactamase (blaOXA-20) and one rifampicin (arr-2) resistance gene in various combinations. In conclusion, the occurrence of A. baumannii isolates producing OXA carbapenemase and 16S rRNA methylase in Norway was related to the worldwide distribution of international clones I and II, and the emergence of novel international clones.

INTRODUCTION

Acinetobacter baumannii is an important opportunistic pathogen that mainly infects critically ill patients in intensive care units (Dijkshoorn et al., 2007). A. baumannii has several innate resistance mechanisms to a number of antibiotics, such as aminopenicillins, first- and second-generation cephalosporins and chloramphenicol.
(Dijkshoorn et al., 2007). Besides this, it has a considerable capacity to acquire mechanisms conferring resistance to broad-spectrum β-lactams, carbapenems, aminoglycosides and fluoroquinolones (Dijkshoorn et al., 2007; Poirel & Nordmann, 2006a). Resistance to carbapenems in A. baumannii is mainly mediated by the acquisition of class D and class B carbapenemase-encoding genes, with blaOXA-23-like being the most frequently identified carbapenemase-encoding gene (Poirel & Nordmann, 2006a). The occurrence of genes encoding aminoglycoside-modifying enzymes, possibly as gene cassettes in class 1 integrons, is the main mechanism of resistance to aminoglycosides (Nemec et al., 2004). In addition, 16S rRNA methylases conferring high-level pan-aminoglycoside resistance have recently been identified in A. baumannii, including OXA-23-producing isolates (Karthikeyan et al., 2010).

Three epidemic clones of A. baumannii, recently named ‘international clones I, II, and III’, have been found to be responsible for several hospital outbreaks globally (Diancourt et al., 2010). A shift in the current A. baumannii population towards international clone II, rather than international clone I, has been reported (Nemec et al., 2008). Multilocus sequence typing (MLST) has also demonstrated the worldwide predominance of two major clonal complexes (CGs), CC92 and CC109 (http://pubmlst.org/abaumannii/). CC92 and CC109 were correlated with international clones II and I, respectively (Mugnier et al., 2010). However, other clones of A. baumannii have also showed a large-scale distribution in geographically distinct regions in the world (Diancourt et al., 2010; Towner et al., 2008). The association between drug resistance and epidemicity in A. baumannii has been established, probably in a mutual escalating arrangement in which acquisition of drug-resistance determinants has facilitated the spread of specific clones and the epidemic capacity of some clones has contributed in the growing emergence of drug resistance (Diancourt et al., 2010). In the present study, we characterized a set of 11 isolates of carbapenem-resistant A. baumannii imported to Norway from other countries with regard to their molecular epidemiology, and their phenotypic and genetic antibiotic-resistance features.

METHODOLOGY

Bacterial isolates. The study included all carbapenem-resistant A. baumannii clinical isolates submitted between 2004 and 2009 to the Reference Centre for Detection of Antimicrobial Resistance in Norway (Table 1). The isolates (n=11) were collected by six different diagnostic microbiology laboratories in Norway from different cultures (blood, pus, respiratory secretions, abdominal cavity fluid and spinal fluid) based on carbapenem resistance according to the guidelines of the Reference Centre of Antimicrobial Resistance. Interestingly, all isolates were derived from patients recently hospitalized abroad (Table 1). Species identification of the isolates was genetically confirmed by partial rpoB (zone 1, 352 bp) and near-complete 16S rRNA gene (1379 bp) sequence analyses (Nemec et al., 2009; Ibrahim et al., 1997).

Molecular epidemiology. MLST was performed according to the scheme described on the Pasteur Institute’s MLST website (http://www.pasteur.fr/recherche/genopole/PB8/ MLST/) (Nemec et al., 2008), and the scheme developed by Burtetal. (2005), with minor modifications (http://pubmlst.org/abaumannii/). Primers gpiKres-F and gpiKres-R, and the published primers rpoD-F2 and rpoD-R2, were used for amplification and sequencing of genes gpi and rpoD, respectively (Supplementary Table S1, available with the online journal) (Park et al., 2009). Sequences were compared with those in A. baumannii MLST databases and analysed using eBURST V3 (http://eburst.mlst.net) under stringent (minimum of six shared alleles) grouping parameters. To differentiate between the two MLST schemes, sequence types (STs) and CCs were designated ST1/CC1 for the Pasteur Institute scheme and ST1P/CC1P for the Burt et al. scheme. Identification of major sequence groups/international clones was done by two multiplex PCRs targeting ompA, csuE, and the blaOXA-23-like sequences (Turton et al., 2007). Full-length sequencing of the blaOXA-23-like was performed using primers OXA-69A and OXA-69B, external to the blaOXA-23-like gene (Héritier et al., 2005). PFGE was performed using Apal-digested genomic DNA, as described by Mugnier et al. (2010). Similarities among the PFGE patterns were calculated by the Dice coefficient method using BioNumerics software (Applied Maths).

Antimicrobial-susceptibility testing. MICs were determined by Etest (bioMérieux) according to the manufacturer’s instructions. Results were interpreted using clinical breakpoints as defined by the European Committee on Antimicrobial Susceptibility Testing, except for tigecycline for which the epidemiological cut-off value (non-wild-type >1 mg L⁻¹) was used (http://www.eucast.org).

Resistance-gene determination. PCR assays were performed to detect the presence of five groups of OXA-carbapenemase-encoding genes (blaOXA-23-like, blaOXA-24-like, blaOXA-51-like, blaOXA-143-like (Woodford et al., 2006; Higgins et al., 2010a)), five metallo-β-lactamase-encoding genes (blaVIM, blaIMP, blaGES, and blaOXA-48) (Woodford et al., 2006) of class 1 integron, were amplified by PCR targeting the open reading frames for tigecycline for which the epidemiological cut-off value (non-wild-type >1 mg L⁻¹) was used (http://www.eucast.org).

DNA sequencing. PCR products were purified directly using ExoSAP-IT (GE Healthcare Bio-Sciences) or from agarose gels using the QIAquick gel extraction kit (Qiagen), according to the manufacturer’s instructions. Sequencing was performed using BigDye 3.1 technology (Applied Biosystems). Sequence analysis and alignments were performed using Lasergene 8 (DNASTAR) and compared to sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov).

RESULTS

Molecular epidemiology

MLST using the Pasteur Institute scheme revealed four different STs (Table 1). eBURST analysis assigned the isolates into CC2P (n=7), CC1P (n=3) and ST15P (n=1). Using
Table 1. Molecular epidemiology of the isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Place of isolation</th>
<th>Date of isolation</th>
<th>Country of export</th>
<th>PFGE Clonal lineage*†</th>
<th>blaOXA-51-like gene</th>
<th>MLST</th>
</tr>
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<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>K12-21</td>
<td>Sørlandet</td>
<td>February 2004</td>
<td>Greece</td>
<td>International clone II</td>
<td>ST184 (1-12-3-2-4-3)</td>
<td>CC92</td>
</tr>
<tr>
<td>K44-35</td>
<td>Vestfold</td>
<td>May 2007</td>
<td>Thailand</td>
<td>International clone II</td>
<td>ST2 (2-2-2-2-2-2)</td>
<td>CC92</td>
</tr>
<tr>
<td>K47-42</td>
<td>Østfold</td>
<td>December 2007</td>
<td>China</td>
<td>International clone II</td>
<td>ST2 (2-2-2-2-2-2)</td>
<td>CC92</td>
</tr>
<tr>
<td>K48-42</td>
<td>Telelab</td>
<td>February 2008</td>
<td>India</td>
<td>International clone I</td>
<td>ST1 (1-1-1-1-5-1-1)</td>
<td>CC1</td>
</tr>
<tr>
<td>K55-13</td>
<td>Haukeland</td>
<td>February 2009</td>
<td>Cyprus</td>
<td>International clone II</td>
<td>ST2 (2-2-2-2-2-2)</td>
<td>CC92</td>
</tr>
<tr>
<td>K55-61</td>
<td>Vestfold</td>
<td>March 2009</td>
<td>India</td>
<td>Sequence group 9</td>
<td>ST94 (1-2-2-1-5-1-1)</td>
<td>CC1</td>
</tr>
<tr>
<td>K57-06</td>
<td>Ullevål</td>
<td>March 2009</td>
<td>India</td>
<td>Sequence group 9</td>
<td>ST94 (1-2-2-1-5-1-1)</td>
<td>CC1</td>
</tr>
<tr>
<td>K58-15</td>
<td>Ullevål</td>
<td>June 2009</td>
<td>Thailand</td>
<td>International clone II</td>
<td>ST2 (2-2-2-2-2-2)</td>
<td>CC92</td>
</tr>
<tr>
<td>K58-19</td>
<td>Østfold</td>
<td>July 2009</td>
<td>Italy</td>
<td>International clone II</td>
<td>ST2 (2-2-2-2-2-2)</td>
<td>CC92</td>
</tr>
<tr>
<td>K61-46</td>
<td>Ullevål</td>
<td>October 2009</td>
<td>Pakistan</td>
<td>International clone II</td>
<td>ST2 (2-2-2-2-2-2)</td>
<td>CC92</td>
</tr>
<tr>
<td>K71-71</td>
<td>Vestfold</td>
<td>December 2009</td>
<td>Pakistan</td>
<td>Sequence group 4</td>
<td>ST15 (6-6-8-2-3-5-4)</td>
<td>CC92</td>
</tr>
</tbody>
</table>

ND, Not determined.

*International clones I, II and III, and PCR-based sequence groups 4 to 8, were defined by diverse combinations of amplicons obtained in the group 1 and group 2 multiplex PCRs as described elsewhere (Turton et al., 2007; Towner et al., 2008; Giannouli et al., 2010).
†PCR-based sequence group 9 showed a novel combination of amplicon ompA in the group 1 multiplex PCR and amplicons csuE and blaOXA-51-like in the group 2 multiplex PCR.

The Bartual MLST scheme, at least ten different STs were identified (Table 1). An incomplete ST, comprising six of the seven housekeeping loci, was assigned to one isolate since PCR assays failed to amplify the gpi fragment in this isolate. eBURST analysis assigned the isolates into CC92B (n=7), CC109B (n=1), ST194B together with incomplete ST194B (n=2), and CC104B (n=1).

All seven isolates of CC2P/CC92B and the isolate of CC1P/CC109B belonged to international clones II and I, respectively (Turton et al., 2007) (Table 1). The two isolates of CC1P/ST194B showed a new combination of amplicons and were considered in this article as PCR-based sequence group 9. The isolate of ST15P/CC104B showed the same combination of amplicons indicative of PCR-based sequence group 4 (Towner et al., 2008).

Analysis of the intrinsic blaOXA-51-like variants showed that all seven isolates of CC2P/CC92B/international clone II and the isolate of CC1P/CC109B/international clone I contained blaOXA-66 and blaOXA-69, respectively. The two isolates of CC1P/ST194B/PCR-based sequence group 9 also contained blaOXA-69, while the isolate of ST15P/CC104B/PCR-based sequence group 4 contained blaOXA-51 (Table 1). PFGE assigned three isolates into one pulsotype (>80% similarity) and two isolates into another pulsotype (100% similarity) (Table 1). The remaining six isolates each revealed a distinct pulsotype.

Antimicrobial-susceptibility testing

All the isolates were multidrug resistant (Table 2). MICs of imipenem and meropenem ranged from 16 to >32 mg l⁻¹ and from 6 to >32 mg l⁻¹, respectively. All isolates were resistant to at least two aminoglycosides, including four isolates showing high-level resistance to the four aminoglycosides tested. All isolates expressed resistance to ciprofloxacin. In addition, 9/11 isolates were resistant to trimethoprim/sulfamethoxazole and 10/11 isolates had MICs above the epidemiological cut-off value (1 mg l⁻¹) for tigecycline. Colistin was the only antibiotic all isolates were susceptible to.

Resistance-gene determination

All the isolates contained the naturally occurring blaOXA-51-like gene (Table 1). Acquired OXA-carbapenemase-encoding
genes were present in all the isolates: blaOXA-23-like (n=9), blaOXA-24-like (n=1) and blaOXA-58-like (n=1). None of the isolates contained blaOXA-143 or metallo-β-lactamase-encoding genes. ISAb1 was detected upstream and downstream of blaOXA-23-like in seven isolates whereas it was present only upstream of blaOXA-23-like in two isolates (Table 2). Sequence analysis of the ISAb1-blaOXA-23-like structure showed a deletion of 7 bp (CTCTTTT) in one of the latter two isolates compared with the corresponding sequence of the other isolate. In the isolate harbouring blaOXA-58-like, an upstream ISAb3-like element and downstream ISAb3 surrounded the blaOXA-58-like gene. No linkage was detected between ISAb1 and the blaOXA-51-like genes or between ISAb3 and the blaOXA-24-like gene.

The 16S rRNA methylase armA gene was detected in the four isolates showing high levels of resistance to all aminoglycosides tested (Table 2). Double mutations in the quinolone-resistance determining regions of GyrA (Ser-83 to Leu) and ParC (Ser-80 to Leu) were identified in all isolates.

Seven isolates were positive for the intI1 gene. However, PCR amplification of the VR of the class 1 integron gave PCR products in only six isolates. No PCR product was obtained from isolate K61–46, presumably due to absence or significant alteration in the 3’CS region. One isolate (K47–42) yielded two amplification products. Sequence analysis of the integrons revealed the presence of six different VRs (Fig. 1).

### DISCUSSION

The worldwide emergence of multidrug- and carbapenem-resistant A. baumannii isolates is of great concern (Dijkshoorn et al., 2007; Poirel & Nordmann, 2006a). In Norway, only one minor outbreak of carbapenem-resistant A. baumannii has been reported (Onarheim et al., 2000).
The occurrence of clinical isolates producing acquired carbapenemases in Norway has been linked with hospitalization abroad (Samuelsen et al., 2009, 2010). In this study, all the carbapenem-resistant isolates of *A. baumannii* were associated with import to Norway. Thus, the study also provided an international perspective of carbapenem-resistant *A. baumannii*.

The results from this study were consistent with those of other studies regarding the predominance of CC25/CC92/ international clone II within the *A. baumannii* global population (Mugnier et al., 2010; Hamouda et al., 2010). The association between this group and the *bla*OXA-66-like cluster of variants has also been observed by Evans et al. (2008). Of note, the clone included four isolates with distinct pulsotypes, which was most likely due to a higher genetic diversity indexed by PFGE (Hamouda et al., 2010).

Only one isolate belonged to the *bla*OXA-69-cluster-positive CClP/CC109/ international clone I (Mugnier et al., 2010; Hamouda et al., 2010; Evans et al., 2008). The other two *bla*OXA-69-positive isolates belonged to CClP/ST194/PCR-based sequence group 9. These two isolates were identical by PFGE and were both obtained in 2009 from two patients who had initially been hospitalized in India. eBURST analysis showed that ST194 was not part of any CC in the PubMLST database (http://pubmlst.org/abaumannii/). However, ST194 (1-15-4-11-4-58-4) was interestingly found to be a single locus variant of an unassigned ST (1-15-4-6-4-58-4) of isolates also obtained from India between 2008 and 2009 (Ko et al., 2010). Nonetheless, the two isolates were both assigned to CClP, suggesting that PCR-based sequence group 9 could represent a subgroup of international clone I.

One isolate, imported from Pakistan, belonged to ST15/ CC104/PCR-based sequence group 4. Previous studies have reported the occurrence of isolates from this group in India, Europe and South America (Towner et al., 2008; Higgins et al., 2010b). Furthermore, ST15 has included multidrug-resistant and carbapenem-resistant isolates, supporting the proposal that this group represents an antimicrobial-resistant novel international clone (www.pasteur.fr/recherche/genopole/PAF8/mlst/Abaumannii.html) (Diancourt et al., 2010; Mugnier et al., 2010).

Resistance to carbapenems in *A. baumannii* has mainly been related to the presence of OXA carbapenemases and linked to ISAba elements (Poirel & Nordmann, 2006a). Seven of our isolates contained the ISAba1-blaOXA-23-like ISAba1 genetic arrangement. The length of PCR products suggested that blaOXA-23-like could be part of Tn0006 in these isolates (Corvec et al., 2007). ISAba1-blaOXA-23-like ISAba1 was present in isolates of international clone II (n=3), international clone I (n=1), PCR-based sequence group 4 (n=1) and PCR-based sequence group 9 (n=2). The occurrence of this resistance structure in isolates of different clonal lineages most likely indicates a successful horizontal transfer. The ISAba1-blaOXA-23-like arrangement, without a downstream ISAba1, was detected in two isolates. Interestingly, ISAba1-blaOXA-23-like showed a 7 bp deletion of CTCTTTT in one of these two isolates, suggesting that blaOXA-23-like could be part of Tn0008 in this isolate (Adams-Haduch et al., 2008). In accordance with other studies, the 16S rRNA methylase-encoding armA gene was detected in three isolates belonging to international clone II (Cho et al., 2009). However, the fourth armA-positive isolate in our study belonged to ST15/CC104/PCR-based sequence group 4, adding more data on the significance of this group with regard to antimicrobial resistance in *A. baumannii*.

The ability of integrons to capture and mobilize gene cassettes has considerably contributed to dissemination of resistance genes among bacteria (Koeleman et al., 2001). Our results were similar to the results of other studies on the geographical distribution of class 1 integrons with VR1 (Europe), VR2 (East and South-East Asia), and VR3, VR4, and VR5 (worldwide) (Nemec et al., 2004; Han et al., 2008; Xu et al., 2008; Adams et al., 2008; Post et al., 2010). To our knowledge, the occurrence of the class 1 integron with only arr-2 and cmrA5 as gene cassettes (VR6) has not been reported before. Similar to what other studies have reported, the deduced amino acid sequences of the class 1 integron-located aacA4 genes in our isolates were consistent with AAC(6')-Ib and not with AAC(6')-Ib-cr (Xu et al., 2008).

In conclusion, this study demonstrated the major role of the highly successful international clones I and II and the supplementary role of other emerging clones in the worldwide spread of multidrug-resistant *A. baumannii* strains. The emergence of epidemic multidrug-resistant *A. baumannii* clones in Norwegian hospitals points to the necessity of a screening programme for patients after hospitalization abroad, and strict infection control regimes to prevent further antimicrobial-resistance selection and subsequent dissemination.

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