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 Sundsfjord^{1,2} and Ørjan Samuelsen¹

Correspondence

Nabil Karah

nabil.karah@uit.no

Ørjan Samuelsen

orjan.samuelsen@unn.no

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 *bla*_{OXA-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 *bla*_{OXA-66} and belong to MLST clonal complexes (CCs) CC2^P (Pasteur Institute scheme) and CC92^B (Bartual scheme), and international clone II. One isolate harboured *bla*_{OXA-69}, and belonged to CC1^P, CC109^B 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: *bla*_{OXA-23}-like (*n*=9), *bla*_{OXA-24}-like (*n*=1) and *bla*_{OXA-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 (*bla*_{OXA-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.

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Abbreviations: CC, clonal complex; MLST, multilocus sequence typing; ST, sequence type; VR, variable region.

The GenBank/EMBL/DDBJ accession numbers for the integron variable region sequences of isolates K44-35 and K71-71 are HQ323750 and HQ323751, respectively.

A table of primer information is available as supplementary material with the online journal.

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 *bla*_{OXA-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 (CCs), 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.

METHODS

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 genotypically 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/PF8/mlst/>) (Nemec *et al.*, 2008), and the scheme developed by Bartual *et al.* (2005), with minor modifications (<http://pubmlst.org/abaumannii/>). Primers *gpi*Kres-F and *gpi*Kres-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 ST^P/CC^P for the Pasteur Institute scheme and ST^B/CC^B for the Bartual scheme. Identification of major sequence groups/international clones was done by two multiplex PCRs targeting *ompA*, *csuE* and *bla*_{OXA-51}-like sequences (Turton *et al.*, 2007). Full-length sequencing of *bla*_{OXA-51}-like was performed using primers OXA-69A and OXA-69B, external to the *bla*_{OXA-51}-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 $>1 \text{ mg l}^{-1}$) 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 (*bla*_{OXA-23}-like, *bla*_{OXA-24}-like, *bla*_{OXA-51}-like, *bla*_{OXA-58}-like and *bla*_{OXA-143}) (Woodford *et al.*, 2006; Higgins *et al.*, 2010a), five metallo- β -lactamase-encoding genes (*bla*_{VIM}, *bla*_{GIM}, *bla*_{IMP} and *bla*_{SPM}, and *bla*_{NDM-1}, in-house designed PCR) (Mendes *et al.*, 2007), six 16S rRNA methylase-encoding genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*) (Doi & Arakawa, 2007; Fritsche *et al.*, 2008) and the class 1 integrase-encoding gene (*intI1*) (Koeleman *et al.*, 2001) (Supplementary Table S1, available with the online journal). In addition, linkage PCRs between the OXA-carbapenemase-encoding genes and insertion sequence elements (*ISAbal*, *ISAbal2* and *ISAbal3*) were performed as described elsewhere (Poirel & Nordmann, 2006b; Corvec *et al.*, 2007). The variable regions (VRs) of class 1 integrons were amplified and sequenced using primers 5'CS and 3'CS, and in-house designed internal primers (Tolman *et al.*, 2007) (Supplementary Table S1, available with the online journal). The occurrence of mutations in the quinolone-resistance determining regions of *gyrA* and *parC* genes was detected as described elsewhere (Vila *et al.*, 1995, 1997).

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 CC2^P ($n=7$), CC1^P ($n=3$) and ST15^P ($n=1$). Using

Table 1. Molecular epidemiology of the isolates

Isolate	Place of isolation	Date of isolation	Country of export	PFGE	Clonal lineage*†	<i>bla</i> _{OXA-51} -like gene	MLST	
							Pasteur Institute scheme	Bartual scheme
K12-21	Sørlandet	February 2004	Greece	A	International clone II	<i>bla</i> _{OXA-66}	ST45 (2-6-2-2-2-2-2) CC2	ST189 (1-12-3-2-2-4-3) CC92
K44-35	Vestfold	May 2007	Thailand	B	International clone II	<i>bla</i> _{OXA-66}	ST2 (2-2-2-2-2-2-2) CC2	ST190 (1-3-3-2-2-52-3) CC92
K47-42	Østfold	December 2007	China	A2	International clone II	<i>bla</i> _{OXA-66}	ST2 (2-2-2-2-2-2-2) CC2	ST191 (1-3-3-2-2-94-3) CC92
K48-42	Telelab	February 2008	India	C	International clone I	<i>bla</i> _{OXA-69}	ST1 (1-1-1-1-5-1-1) CC1	ST192 (10-53-4-11-4-95-5) CC109
K55-13	Haukeland	February 2009	Cyprus	D	International clone II	<i>bla</i> _{OXA-66}	ST2 (2-2-2-2-2-2-2) CC2	ST193 (1-35-3-2-2-3-3) CC92
K55-61	Vestfold	March 2009	India	E	Sequence group 9	<i>bla</i> _{OXA-69}	ST94 (1-2-2-1-5-1-1) CC1	ST194 (1-15-4-11-4-58-4) singleton
K57-06	Ullevål	March 2009	India	E	Sequence group 9	<i>bla</i> _{OXA-69}	ST94 (1-2-2-1-5-1-1) CC1	Incomplete ST194 (1-15-4-11-4-ND-4) singleton
K58-15	Ullevål	June 2009	Thailand	F	International clone II	<i>bla</i> _{OXA-66}	ST2 (2-2-2-2-2-2-2) CC2	ST195 (1-3-3-2-2-96-3) CC92
K58-19	Østfold	July 2009	Italy	A3	International clone II	<i>bla</i> _{OXA-66}	ST2 (2-2-2-2-2-2-2) CC2	ST118 (1-3-3-2-2-3-3) CC92
K61-46	Ullevål	October 2009	Pakistan	G	International clone II	<i>bla</i> _{OXA-66}	ST2 (2-2-2-2-2-2-2) CC2	ST137 (1-3-3-2-2-12-3) CC92
K71-71	Vestfold	December 2009	Pakistan	H	Sequence group 4	<i>bla</i> _{OXA-51}	ST15 (6-6-8-2-3-5-4) CC with ST84	ST103 (12-17-12-1-29-3-39) CC104

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 *bla*_{OXA-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 CC92^B ($n=7$), CC109^B ($n=1$), ST194^B together with incomplete ST194^B ($n=2$), and CC104^B ($n=1$).

All seven isolates of CC2^P/CC92^B and the isolate of CC1^P/CC109^B belonged to international clones II and I, respectively (Turton *et al.*, 2007) (Table 1). The two isolates of CC1^P/ST194^B showed a new combination of amplicons and were considered in this article as PCR-based sequence group 9. The isolate of ST15^P/CC104^B showed the same combination of amplicons indicative of PCR-based sequence group 4 (Towner *et al.*, 2008).

Analysis of the intrinsic *bla*_{OXA-51}-like variants showed that all seven isolates of CC2^P/CC92^B/international clone II and the isolate of CC1^P/CC109^B/international clone I contained *bla*_{OXA-66} and *bla*_{OXA-69}, respectively. The two isolates of CC1^P/ST194^B/PCR-based sequence group 9 also contained *bla*_{OXA-69}, while the isolate of ST15^P/CC104^B/PCR-based

sequence group 4 contained *bla*_{OXA-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 *bla*_{OXA-51}-like gene (Table 1). Acquired OXA-carbapenemase-encoding

Table 2. Phenotypic and molecular resistance characteristics of the isolates

Isolate	Susceptibility pattern (MICs in mg l ⁻¹)										Genetic structures flanking the OXA genes	16S rRNA methylase
	IPM	MEM	CIP	AMK	GEN	TOB	NET	SXT	TGC	CST		
K12-21	32	6	>32	6	24	8	>256	2	0.8	0.38	ISAb3-like- <i>bla</i> _{OXA-58} -like-ISAb3	Negative
K44-35	16	32	>32	24	16	24	>256	>32	3	0.75	ISAb1- <i>bla</i> _{OXA-23} -like-ISAb1	Negative
K47-42	>32	>32	>32	>256	>256	>256	>256	>32	2	0.19	ISAb1- <i>bla</i> _{OXA-23} -like	<i>armA</i>
K48-42	>32	>32	>32	64	32	1	4	32	3	0.5	ISAb1- <i>bla</i> _{OXA-23} -like-ISAb1	Negative
K55-13	>32	>32	>32	48	48	48	>256	>32	16	0.75	<i>bla</i> _{OXA-24} -like	Negative
K55-61	32	>32	32	>256	>1024	64	6	>32	2	0.5	ISAb1- <i>bla</i> _{OXA-23} -like-ISAb1	Negative
K57-06	>32	>32	>32	>256	>1024	48	6	>32	2	0.38	ISAb1- <i>bla</i> _{OXA-23} -like-ISAb1	Negative
K58-15	>32	≥32	>32	>256	>1024	>256	>256	1	4	0.38	ISAb1- <i>bla</i> _{OXA-23} -like-ISAb1	<i>armA</i>
K58-19	>32	>32	>32	>256	>1024	>256	>256	>32	2	1	ISAb1- <i>bla</i> _{OXA-23} -like-ISAb1	<i>armA</i>
K61-46	32	>32	>32	192	3	1.5	8	>32	6	0.38	ISAb1- <i>bla</i> _{OXA-23} -like	Negative
K71-71	32	>32	>32	>256	>1024	>256	>256	>32	3	0.19	ISAb1- <i>bla</i> _{OXA-23} -like-ISAb1	<i>armA</i>

AMK, Amikacin; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; NET, netilmicin; SXT, trimethoprim/sulfamethoxazole; TGC, tigecycline; TOB, tobramycin.

genes were also present in all the isolates: *bla*_{OXA-23}-like ($n=9$), *bla*_{OXA-24}-like ($n=1$) and *bla*_{OXA-58}-like ($n=1$). None of the isolates contained *bla*_{OXA-143} or metallo- β -lactamase-encoding genes. *ISAb1* was detected upstream and downstream of *bla*_{OXA-23}-like in seven isolates whereas it was present only upstream of *bla*_{OXA-23}-like in two isolates (Table 2). Sequence analysis of the *ISAb1*-*bla*_{OXA-23}-like structure showed a deletion of 7 bp (CTCTTTT) in one of the latter two isolates compared with the corresponding

sequences of the other isolate. In the isolate harbouring *bla*_{OXA-58}-like, an upstream *ISAb3*-like element and downstream *ISAb3* surrounded the *bla*_{OXA-58}-like gene. No linkage was detected between *ISAb1* and the *bla*_{OXA-51}-like genes or between *ISAb3* and the *bla*_{OXA-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).

Fig. 1. VRs of the detected class 1 integrons.

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 CC2^P/CC92^B/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 CC1^P/CC109^B/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 CC1^P/ST194^B/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^B was not part of any CC in the PubMLST database (<http://pubmlst.org/abaumannii/>). However, ST194^B (1-15-4-11-4-58-4) was interestingly found to be a single locus variant of an unassigned ST^B (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 CC1^P, suggesting that PCR-based sequence group 9 could represent a subgroup of international clone I.

One isolate, imported from Pakistan, belonged to ST15^P/CC104^B/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^P 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/PF8/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 IS*Aba* elements (Poirel & Nordmann, 2006a). Seven of our isolates contained the IS*Aba1*-*bla*_{OXA-23}-like-IS*Aba1* genetic arrangement. The length of PCR products suggested that *bla*_{OXA-23}-like could be part of Tn2006 in these isolates (Corvec *et al.*, 2007). IS*Aba1*-*bla*_{OXA-23}-like-IS*Aba1* 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 IS*Aba1*-*bla*_{OXA-23}-like arrangement, without a downstream IS*Aba1*, was detected in two

isolates. Interestingly, IS*Aba1*-*bla*_{OXA-23}-like showed a 7 bp deletion of CTCTTTT in one of these two isolates, suggesting that *bla*_{OXA-23}-like could be part of Tn2008 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^P/CC104^B/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 *cm1A5* 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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