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Genetic diversity of carbapenem-resistant isolates of *Acinetobacter baumannii* in Europe

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ABSTRACT

In total, 96 carbapenem-resistant isolates of *Acinetobacter baumannii* were obtained from 25 hospitals in 17 European countries. Imipenem MICs ranged from <4 to 128 mg/L on retesting by Etest, with MICs ≥16 mg/L being associated with the carriage of genes encoding at least one other class D carbapenemase in addition to the intrinsic OXA-51-like enzyme. Molecular typing results obtained by random amplified polymorphic DNA analysis, followed by pulsed-field gel electrophoresis (PFGE) of *Apa*I-digested chromosomal DNA, were highly congruent, with 17 different PFGE types being delineated at a cut-off similarity level of 85%. With few exceptions, multiple isolates from a single hospital belonged to the same PFGE type. Seven sequence groups were identified among the 96 *A. baumannii* isolates, with the majority of isolates (n = 81) belonging to the previously defined sequence groups 1 and 2, which each included eight PFGE types. These two multinational lineages included the previously defined European clones II and I, respectively, but the problem of resistant *A. baumannii* in Europe appeared not to be confined solely to these two European clones. Rather, two broader lineages of carbapenem-resistant *A. baumannii* now seem to be spreading throughout Europe.

Keywords *Acinetobacter baumannii*, carbapenem resistance, epidemiology, Europe, sequence groups, typing

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INTRODUCTION

Antimicrobial resistance is causing increasing concern worldwide, with many of the major resistance problems being caused by nosocomial pathogens present in the hospital environment. Intensive antibiotic use in hospitals potentiates these resistance problems, which are further compounded by the ever-increasing immunosuppression that comes with modern diagnostic and therapeutic modalities, and the opportunities for cross-infection that exist in modern, busy, often over-crowded and under-staffed hospitals. Against this background, four study groups of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) formulated the ARPAC (Antibiotic Resistance, Prevention and Control) project to investigate what measures were being attempted to control antimicrobial resistance among key 'alert' organisms causing infections in hospitals at a pan-European level [1].

One of the key multiresistant 'alert' organisms currently posing major challenges to hospitals in Europe is *Acinetobacter baumannii*. This organism, and its close relatives, can cause a wide range of nosocomial infections, especially in immunocompromised patients, but their most important role is as a cause of nosocomial pneumonia, particularly in intensive care units [2–4]. Many nosocomial isolates of *Acinetobacter* are multiresistant, meaning that infections caused by these organisms are difficult to treat, even with combination therapy [4]. Moreover, these organisms often

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cause outbreaks of infection [2–4] and can survive for long periods in the hospital environment, even in dry conditions [5].

Carbapenems have been the mainstay of treatment for *Acinetobacter* infections for the past decade, and still remain active in many centres, but reports of carbapenem resistance have now accumulated worldwide [6,7]. Most recently, significant problems have been caused by multiresistant strains of *A. baumannii* in hospitals in Europe and the USA following repatriation of military patients from the ongoing conflict in Iraq [8,9], with very few therapeutic options remaining for the treatment of patients infected by such multiresistant strains. The emergence of carbapenem resistance among strains of *A. baumannii* is now recognised as a major problem that warrants microbiological and epidemiological interventions [10]. Accordingly, the aim of the present study was to gain an insight into the population structure of carbapenem-resistant strains of *A. baumannii* causing nosocomial infections in hospitals across Europe.

MATERIALS AND METHODS

Bacterial isolates

The ARPAC project [1] collected data concerning key 'alert' organisms, including multiresistant *A. baumannii*, from 169 hospitals in 32 European countries between 2001 and 2004. Hospitals that reported encountering carbapenem-resistant strains of *Acinetobacter* spp. were contacted during 2004 and asked to submit examples of these strains where these were still available. In total, 84 isolates of *Acinetobacter* were obtained from 22 ARPAC-participating hospitals; a further 16 isolates from the same period were obtained from three non-ARPAC hospitals to obtain a broader representation. Also included in the study were standard reference strains of European clones I (RUH 2034), II (RUH 134) and III (RUH 5875), defined previously by amplified fragment length polymorphism (AFLP) analysis [11,12] (gift of L. Dijkshoorn, Leiden University Medical Center, The Netherlands).

Isolates were originally identified as *Acinetobacter* spp. at the submitting laboratories by a variety of routine microbiological methods. Identification of isolates as members of the *Acinetobacter calcoaceticus–A. baumannii* (*Acb*) complex was confirmed on receipt by tRNA spacer fingerprinting [13], followed by the use of a multiplex PCR [14] to demonstrate the presence of a gene encoding an OXA-51-like enzyme, which is considered to be an enzyme that is intrinsic and unique to *A. baumannii* [15,16]. The same multiplex PCR was used to simultaneously demonstrate the presence of OXA-23-like, OXA-24-like and OXA-58-like enzymes. All isolates were tested for susceptibility to imipenem using Etests (AB Biodisk, Solna, Sweden) as recommended by the manufacturer.

Molecular typing

Random amplified polymorphic DNA analysis (RAPD). Initial RAPD analysis of the isolates was performed using primer DAF4 as described by Grundmann *et al.* [17], with analysis of RAPD products on agarose 1.5% w/v gels in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). A 100-bp DNA ladder (GE Healthcare Life Sciences, Little Chalfont, UK) was included as a standard for gel normalisation purposes in the left- and right-hand lanes of each gel, and at least once every six lanes. Following staining with ethidium bromide, the gel images were saved as TIFF files using a GAS-9201 UVIsave image analysis system (UVItec, Cambridge, UK). Gel analysis was performed using BioNumerics v.2.0 (Applied Maths, Sint-Martins-Latem, Belgium). Similarity was calculated using the Dice coefficient with an optimisation of 0.5%, a tolerance of 1.5%, and the unweighted pair-group method using arithmetic averages (UPGMA). It has been shown previously [18] that good discrimination is achieved between genetically unrelated groups of *Acinetobacter* spp. using a similarity cut-off value of 72%. Isolates that clustered together with a similarity of >72% were therefore considered to belong to the same RAPD type.

Pulsed-field gel electrophoresis (PFGE). Isolates were typed by PFGE using a method based on that described by Bannerman *et al.* [19]. In brief, intact genomic DNA was prepared in agarose plugs and then digested with *Apa*I (Promega, Southampton, UK). DNA fragments were separated on agarose 1% w/v gels in 0.5× TBE buffer at 14°C in a CHEF DRII apparatus (Bio-Rad, Hemel Hempstead, UK) at 200 V, with pulses ramped from 5 to 13 s for 20 h. An *Apa*I digest of genomic DNA from *A. baumannii* strain RUH 2034 was included as a standard for gel normalisation purposes in the left- and right-hand lanes of each gel, and at least once every six lanes. Gels were stained with ethidium bromide, saved as TIFF files and analysed using BioNumerics v.2.0 as described above. Isolates that clustered together with a similarity of >85% were considered to belong to the same PFGE type.

Identification of PCR-based sequence groups

Two multiplex PCRs, designed to selectively amplify group 1 or group 2 alleles of the *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ genes, were performed as described by Turton *et al.* [20], except that each reaction used a Ready-to-Go PCR Bead (GE Healthcare Life Sciences) containing pre-formulated PCR buffer, dNTPs and *Taq* polymerase in a final reaction volume of 25 μL.

RESULTS

Identification of isolates

In total, the present study collected 100 *Acinetobacter* isolates from 25 hospitals in 17 European countries (Table 1). All of these isolates were identified as members of the *Acb* complex according to tRNA spacer fingerprinting. However, this technique has difficulty in distinguishing among the closely related genomic species contained within the *Acb* complex, and four isolates were

Table 1. Geographical location of hospitals submitting carbapenem-resistant isolates of *Acinetobacter baumannii* (n = 96) showing clusters according to pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analysis, and OXA-type enzymes

Country	Hospital	No. of isolates	PFGE type	RAPD types	OXA enzymes[a]
Spain	A	2	I	16	OXA-24-like
Poland	B	6	II	13, 14	
		2	VII	4	OXA-23-like
	C	6	VIII[b]	6	
		1	XV	12	
		3	VII	4	
	D	1	VIII[b]	6	
		1	XVI[c]	15	OXA-23-like
		1	XVII	23	
	E[d]	1	VIII[b]	6	
		1	XVI[c]	15	
		1	XVII	23	
	F	9	XVI	I5	
Czech Republic	G	1	III	15	
Slovakia	H	2	III	10	
Germany	I	1	IV	11	OXA-58-like
		1	X	19	
	J[e]	1	XI	21	OXA-58-like
Croatia	K	16	V	1, 2, 8	
Norway	L	1	VI	9	OXA-58-like
Bulgaria	M	2	VII	1	OXA-23-like
	N	5	IX	1	OXA-23-like
Slovenia	O	1	VII	3	OXA-23-like
Turkey	P	2	X	19, 20	OXA-58-like
Estonia	Q	3	XII	24	
Austria	R	1	XIII	17	
Portugal	S	7	XIV	22	OXA-24-like
Denmark	T	1	XV	12	OXA-58-like
UK	U	4	XVI[c]	15	OXA-23-like
Greece	V	5	IV	11, 13	OXA-58-like
	W	2	IV	7, 18	OXA-58-like
	X	2	IV	7	OXA-58-like
		2	XVI[c]	15	OXA-23-like
The Netherlands	Y[d]	1	X	19	OXA-23-like

[a]Presence of gene encoding an OXA-type carbapenemase in cluster in addition to the intrinsic OXA-51-like enzyme.
[b]Formed a PFGE cluster with the reference strain of European clone I [11,12].
[c]Formed a cluster with the reference strain of European clone II [11,12].
[d]One additional isolate from this hospital lacked a gene encoding an OXA-51-like gene and was discarded.
[e]Two additional isolates from this hospital lacked a gene encoding an OXA-51-like gene and were discarded.

revealed by PCR to lack a gene encoding an OXA-51-like carbapenemase, which is an enzyme that is considered to be intrinsic and unique to *A. baumannii* [15,16]. These four isolates were therefore not studied further.

Carbapenem resistance of isolates

Although all of the remaining 96 isolates were considered by the submitting laboratories to be resistant to carbapenems, Fig. 1 shows that, according to Etests, the distribution of imipenem MICs for these isolates varied from <4 to 128 mg/L, with no clear cut-off between 'resistant' and 'susceptible' isolates. As mentioned above, each of these isolates harboured a gene encoding an OXA-51-like class D β-lactamase; however, the multiplex PCR used to identify the OXA-51-like gene also revealed that 26 (92.9%) of the 28 isolates with an imipenem MIC of ≥16 mg/L (which would be regarded as resistant according to CLSI breakpoint criteria [21]) also harboured a gene encoding either an additional

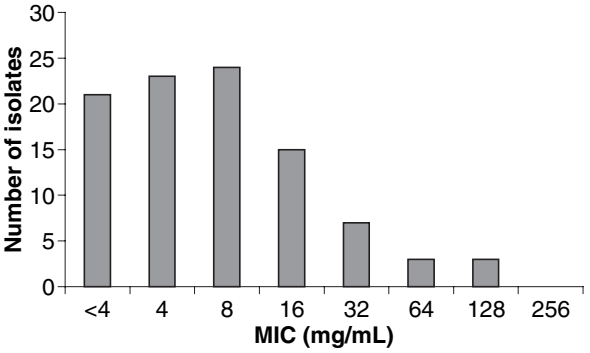

Fig. 1. Distribution of imipenem MICs among 96 clinical isolates of *Acinetobacter baumannii* considered to be carbapenem-resistant by 26 hospitals in 17 European countries.

OXA-23-like (n = 6), OXA-24-like (n = 5) or OXA-58-like (n = 15) enzyme, compared with only five (7.3%) (two OXA-23-like and three OXA-58-like enzymes) of the 68 isolates with an imipenem MIC of <16 mg/L. The geographical and clonal distribution of the isolates that produced additional OXA enzymes is shown in Table 1.

Typing of isolates by RAPD and PFGE

Initial RAPD analysis using the DAF4 primer and a previously defined similarity cut-off value of 72% revealed that the 96 *A. baumannii* isolates belonged to 24 RAPD types. Subsequent PFGE analysis based on *Apa*I digestion of total chromosomal DNA revealed 17 PFGE types, with some PFGE types encompassing more than one RAPD type. With few exceptions (hospitals B, C, D, E, I and X), multiple isolates from a single hospital belonged to the same PFGE type (Table 1). There were only three RAPD types (nos 1, 13 and 15) that corresponded to more than one PFGE type (Table 1). The reference strain of European clone I clustered (using an 85% PFGE cut-off level) with two clinical isolates from Poland. Similarly, the reference strain of European clone II formed a cluster at the 85% PFGE cut-off level with four clinical isolates from the UK, two isolates from Poland and two isolates from Greece (Table 1). The reference strain of European clone III had a PFGE profile that did not cluster with any of the clinical isolates in the present study. Table 2 summarises the inter-relationships of PFGE and RAPD types.

Table 2. Relationship between sequence groups, pulsed-field gel electrophoresis (PFGE) types and randomly amplified polymorphic DNA (RAPD) types for 96 clinical isolates of *Acinetobacter baumannii* from European hospitals

Sequence group[a]	PFGE types	RAPD types	Geographical origin of isolates
1 (n = 30)	I	16	Spain
	II	13, 14	Poland
	III	10, 15	Czech Republic, Slovakia
	IV	11, 13, 18	Germany, Greece
	X	19	Germany
	XV	12	Poland
	XVI[b]	15	Greece, Poland, UK
	XVII	23	Poland
2 (n = 51)	IV	7	Greece
	V	1, 2, 8	Croatia
	VI	9	Norway
	VII	1, 3, 4	Bulgaria, Poland, Slovenia
	VIII[c]	6	Poland
	IX	1	Bulgaria
	XII	24	Estonia
	XVII	5	Poland
3 (n = 1)	XI[d]	21	Germany
4 (n = 10)	X	19, 20	The Netherlands, Turkey
	XIV	22	Portugal
5 (n = 2)	XIII	17	Austria
	XV	12	Denmark
6 (n = 1)	IV	7	Greece
7 (n = 1)	XII	24	Estonia

[a]Sequence groups 1, 2 and 3 correspond to those defined by Turton *et al.* [20].
[b]Formed a PFGE cluster with the reference strain of European clone II [11,12].
[c]Formed a PFGE cluster with the reference strain of European clone I [11,12].
[d]Did not form a PFGE cluster with the reference strain of European clone III [12].

Sequence groups

Based on two multiplex PCRs designed to detect allele variations in the *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ genes, seven different sequence groups were identified among the 96 *A. baumannii* isolates. Table 2 summarises their relationship with the PFGE and RAPD types. The majority of isolates (n = 81) belonged to previously defined sequence groups 1 and 2, which each included eight PFGE types (Table 2). As described previously, these two multinational lineages also included European clones II and I, respectively [20]. Sequence group 3 contained European clone III and one other PFGE type (one isolate). The remaining 14 isolates belonged to four other variant sequence groups, defined according to new combinations of amplicons obtained in the two separate multiplex PCRs (Table 3). Interestingly, five PFGE types (IV, X, XII, XV and XVII) were represented in more than one sequence group (Table 2).

DISCUSSION

Of 169 European hospitals in 32 European countries that provided data to ARPAC [1], 130 reported that they had encountered carbapenem-resistant isolates of *Acinetobacter*, ranging from sporadic resistant isolates to an endemic/epidemic situation (ARPAC, unpublished data). Of the 22 hospitals that provided isolates for the present study, the majority were located in eastern, central or southeastern countries of Europe (Table 1), which may indicate particular problems with epidemic multiresistant strains of *A. baumannii* in these areas. Data concerning carbapenem-resistant strains of *A. baumannii* in these countries are scarce, but it has been reported that such strains are prevalent in hospitals in Greece [22] and Poland [23]. Sporadic isolates from hospitals in countries such as Denmark, Norway and The Netherlands may be a consequence

Table 3. New combinations of amplicons obtained in the multiplex PCRs used to define variant sequence groups

Variant sequence group designation	Group 1 PCR			Group 2 PCR		
	csuE 702 bp	$bla_{\text{OXA-51}}$ 559 bp	*ompA* 355 bp	*csuE* 580 bp	*ompA* 343 bp	$bla_{\text{OXA-51}}$ 162 bp
4	–	+	+	–	–	–
5	–	–	+	–	–	–
6	+	–	–	–	+	+
7	–	–	+	+	–	–

of travellers returning from countries where resistant strains are more prevalent. The distribution of imipenem MICs for the 96 isolates of *A. baumannii* varied from <4 to 128 mg/L, with no clear cut-off between 'resistant' and 'susceptible' isolates, which may indicate the existence of a range of different resistance mechanisms.

The precise mechanism(s) of carbapenem resistance in these isolates was not the focus of the present investigation, but the study revealed that 92.9% of the isolates with an imipenem MIC of ≥16 mg/L also harboured a gene encoding either an additional OXA-23-like, OXA-24-like or OXA-58-like enzyme, as compared with only 7.3% of the isolates with an imipenem MIC of <16 mg/L. While the genes encoding an OXA-24-like enzyme were confined to two molecular types from Spain and Portugal, the genes encoding the OXA-23-like and OXA-58-like genes were more widely distributed among a number of different types. This is in agreement with previous reports that genes encoding OXA-23-like and OXA-58-like enzymes have greater mobility than genes encoding OXA-24-like enzymes. In particular, OXA-58 has been associated with carbapenem resistance in isolates of *A. baumannii* in Greece, southern Europe, the Balkans and central Turkey [22,24], while OXA-24-like enzymes (OXA-40) have been associated particularly with isolates from the Iberian peninsula [25,26]. However, as a general observation, *A. baumannii* isolates from Europe that exhibit higher levels of carbapenem resistance seemed to express at least one other class D OXA-type carbapenemase in addition to the intrinsic OXA-51-like enzyme.

The primary aim of the present study was to gain an insight into the population structure of carbapenem-resistant isolates of *A. baumannii* causing nosocomial infections in hospitals across Europe. The present study revealed 17 PFGE types among the 96 isolates examined, with the majority (84%) of these belonging to two multinational lineages, defined on the basis of sequence variations among different alleles of the *ompA*, *csuE* and $bla_{\text{OXA-51}}$-like genes. Previous studies of nosocomial outbreaks of infection caused by *A. baumannii* have used AFLP analysis to identify three pan-European 'clones' [11,12]. Unfortunately, the AFLP technique is only used routinely in a single laboratory in The Netherlands, and it is not clear how the results obtained by AFLP compare with those obtained by the more widely used reference standard technique of PFGE. Sequence groups 1 and 2 were found to include AFLP European clones II and I, respectively, as also reported by Turton *et al.* [20], and it seems that these two sequence groups may represent two broader lineages of resistant *A. baumannii* that are spreading in Europe. In support of this hypothesis, previous studies have also suggested that the problem of multiresistant *A. baumannii* is not confined solely to the well-known European clones identified by AFLP. Thus, an analysis by AFLP of *A. baumannii* isolates from 46 hospitals throughout the UK in 1999–2001 revealed the presence of new outbreak-associated genotypes [27], and a study of endemic *A. baumannii* strains in Sofia, Bulgaria revealed that only a single isolate belonged to a previously identified European AFLP clone [28].

The results obtained using the two group-specific multiplex PCRs were remarkably congruent with the PFGE results. Some PFGE types were represented in more than one sequence group, but this is perhaps not surprising, as the PFGE type is based on an analysis of restriction sites throughout the entire genome, whereas the sequence group depends on sequence variations in the alleles of only three genes. What is perhaps more remarkable is the overall stability of the sequence groups in these carbapenem-resistant isolates from throughout Europe, which suggests that these multinational lineages must have specific features that contribute to their successful dissemination and overall pathogenicity. PFGE enabled greater discrimination within the sequence groups, and this 'micro-variation' can be used to investigate outbreaks and to compare data among laboratories. Similarly, despite well-known limitations, RAPD analysis produced results that were broadly congruent with those obtained by PFGE. With few exceptions, multiple isolates from a single hospital belonged to the same PFGE and RAPD types.

In conclusion, the majority (84%) of carbapenem-resistant isolates from European hospitals belonged to two multinational sequence groups, which each included eight PFGE types. Carbapenem resistance in these isolates was usually associated with the expression of at least one other OXA-type carbapenemase in addition to the OXA-51-like enzyme that is intrinsic to *A. baumannii*. Although the present study identified two of the European clones defined previously on the

basis of AFLP analysis, the problem of resistant *A. baumannii* in Europe may not be confined solely to these two AFLP clones, and two broader lineages of resistant *A. baumannii* may be spreading throughout Europe. Several other variant sequence groups were identified, and comparison of these less successful lineages with the predominant sequence groups may enable new insights into the reasons for successful dissemination and/or pathogenicity.

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