Epidemiol. Infect. (2009), **137**, 174–180. © 2008 Cambridge University Press
doi:10.1017/S0950268808000824 Printed in the United Kingdom

Genetic relatedness of multidrug-resistant *Acinetobacter baumannii* endemic to New York City

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(*Accepted 2 May 2005; first published online 27 May 2008*)

SUMMARY

Multidrug-resistant isolates of *Acinetobacter baumannii* from New York City generally belong to one of three ribotypes. To assess the accuracy of ribotyping, the relatedness of representative isolates was further assessed by rep-PCR, pulsed-field gel electrophoresis (PFGE), and DNA sequencing of five genes potentially associated with antimicrobial resistance (*ampC*, *ompA*, *adeB*, *adeR*, and *abeM*). The isolates fell into several major groups. The first group shared the same ribotype and had common mutations affecting OmpA, AdeR, and AbeM, but consisted of two subtypes with distinctive rep-PCR and PFGE patterns and *ampC* mutations. The second and third groups shared common alterations in OmpA, AdeR, and AbeM, but had distinct ribotype, rep-PCR, and PFGE patterns. The resistant isolates were unrelated to the β-lactam susceptible isolates. Many of the resistant strains shared OmpA and AdeB patterns observed in several European clonal groups. Further development of a multilocus sequencing typing scheme will help determine if multidrug-resistant isolates from diverse geographic areas are indeed ancestrally related.

INTRODUCTION

Acinetobacter baumannii is an increasingly recognized nosocomial pathogen. Because of its ability to survive on environmental surfaces and its intrinsic resistance to many antimicrobial agents, *A. baumannii* has become a challenging opportunistic pathogen in many medical centres worldwide. Genetic fingerprinting of *A. baumannii* is important for infection control studies and epidemiological investigations, and several methods have been employed for this purpose. Pulsed-field gel electrophoresis (PFGE) is often considered the most discriminative method for genotyping *A. baumannii* [1, 2], but it is labour intensive, difficult for inter-laboratory comparisons, and may not be the most appropriate method to examine strains from geographically diverse areas. Amplified fragment length polymorphism analysis has been used to identify *Acinetobacter* at the species level and to effectively genotype clinical isolates from several geographic areas in Europe [3, 4]. Two PCR-based fingerprinting methods, random amplified polymorphic DNA (RAPD) analysis and repetitive extragenic palindromic-sequence based PCR (rep-PCR), have also been used to type *A. baumannii* [3, 5, 6]. Inter-laboratory comparison is possible using standardized methods and reagents [7], however, multiple primers may be needed for optimal discrimination by RAPD analysis [3]. Multilocus PCR with electrospray ionization mass spectrometry has yielded results comparable with PFGE and has accurately genotyped *Acinetobacter* spp. from diverse geographic regions [8, 9], however, the methodology may not be available for many laboratories. A multilocus sequence typing

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(MLST) scheme, using seven housekeeping genes, has been developed as a typing method for *A. baumannii* [10]. The results with MLST have been comparable to PFGE, and MLST allows for the generation of an easily accessible databank necessary for inter-laboratory comparisons. However, the cost of sequencing seven genes per isolate may limit the usefulness of MLST for some laboratories. Sequencing of a single gene (*adeB*) has also been proposed as a method for typing *A. baumannii* [11]. Last, ribotyping has been used to identify species [12] and to characterize isolates of *A. baumannii* from different regions [13–15], but it has been reported to be not as discriminatory as PFGE [2].

In prior surveillance studies involving hospitals in Brooklyn, NY, USA [13, 14], most multidrug-resistant isolates of *A. baumannii* belonged to a small number of ribotypes but owing to uncertainty of the discriminatory power of this method, two aims were established for this investigation. First, the genetic relatedness of isolates belonging to the predominant ribotypes was further investigated by rep-PCR and PFGE. Second, the sequences of five chromosomal genes possibly contributing to antimicrobial resistance were assessed as a potential typing strategy. For comparative purposes, a group of susceptible isolates was also included for genotypic analysis.

MATERIALS AND METHODS

Bacterial isolates

A total of 43 clinical isolates of *A. baumannii*, obtained from Brooklyn-wide surveillance studies [16], were examined. These isolates were selected based on their susceptibility patterns to various classes of antimicrobial agents. Nineteen isolates belonged to one ribotype (28-5), and 12 to a second ribotype (35-2), which together accounted for $>80\%$ of carbapenem-resistant isolates [13, 17]. Five fell into a third ribotype that represented a further 10% of resistant isolates. The remaining seven isolates (of unknown ribotype) were selected based on their susceptibility to most β-lactam antibiotics.

Genetic fingerprinting

Isolates were ribotyped using the Riboprinter™ Microbial Characterization System (Qualicon, Wilmington, DE, USA) using *Eco*RI as the restriction enzyme, according to the manufacturer's recommendations. Isolates were considered related if they had a similarity coefficient of $\geqslant 90\%$; results were verified by visual inspection. Isolates were further characterized using rep-PCR with the ERIC-2 primer, as previously described [7]. Reactions were carried out using Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and established PCR conditions [7]. Isolates were considered related if there was a 0–1 band difference. Isolates were also analysed by PFGE using *Sma*I as the restriction enzyme, according to previously described methods [2], and interpreted using standard definitions [18].

DNA sequencing studies

An ongoing study involves the examination and expression of β-lactamases, porins, and efflux pumps in these isolates of *A. baumannii*. As part of this study, five genes (*ampC*, *ompA*, *adeR*, *adeB*, and *abeM*) were investigated. The gene encoding the chromosomal cephalosporinase AmpC was amplified using previously described primers and PCR conditions [19]. PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and DNA sequenced using the automated fluorescent dye terminator sequencing system (Applied Biosystems, Foster City, CA, USA). Results were compared to the gene encoding ADC-1 [20; GenBank accession no. AJ009979].

Four other genes that may contribute to antimicrobial resistance were also examined. The gene encoding for OmpA, a major porin in the *A. baumannii* cell membrane, was analysed using the following primers: ompA-F 5′-GGCTTGAGCTT-GAACAACAA-3′ and ompA-R 5′-TGTTCAGCT-AAAACAGTACGGC-3′; sequences were compared to GenBank no. AY485227. The gene encoding for AdeR, a regulatory gene for the *adeABC* complex, was amplified with the following primers: adeR-F 5′-AGCGTATGATGAGTTGAAGCA-3′ and adeR-R 5′-AATCCAGCCTTTTTCAATCG-3′; sequencing results were compared to GenBank no. CT025814. An internal 1142 bp product of *adeB* was amplified with the following primers: adeB-F 5′-CGGAAGGCATGGAGTTTAGT-3′ and adeB-R 5′-CTGCCATTGCCATAAGTTCA-3′. This segment includes 526 bp of the *adeB* sequence previously used for typing *A. baumannii* [11]. Sequences for *adeB* were compared to strain BM4454 (GenBank no. AF370885), as previously described [11]. Finally, the gene encoding for AbeM, an efflux pump protein

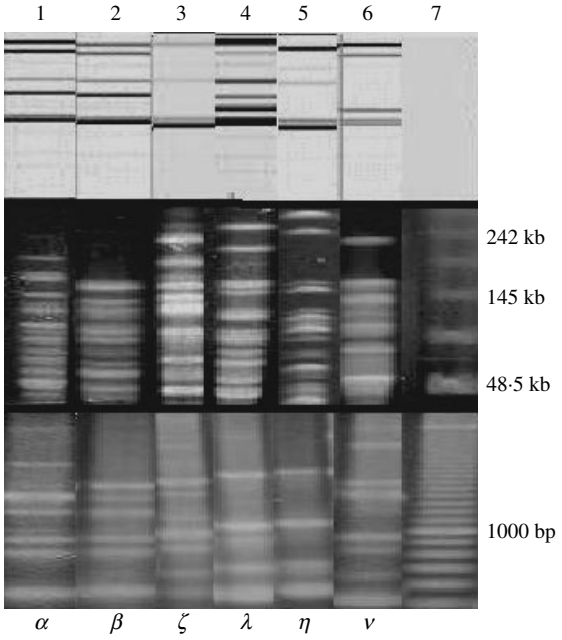

Fig. 1. Ribotyping (top), PFGE (middle), and rep-PCR (bottom) patterns of representative isolates of *A. baumannii*. Lanes 1–6 are isolates belonging to the α, β, ζ, λ, η, and ν groups, respectively; lane 7 has molecular-weight markers.

belonging to the multidrug and toxic compound extrusion family, was amplified using the following primers: abeM-F 5′-TGCAACGCAGTTTCATTTTT-3′ and abeM-R 5′-CGATGTTTCATCGGCTTTTT-3′. Sequences for this gene were compared to GenBank no. AB204810.

Isolates were initially grouped according to ribotype (numerical designation) and then grouped according to rep-PCR type and PFGE group. Isolates related by ribotyping, rep-PCR, and PFGE were designated into a final group, identified by Greek letters.

Nucleotide sequence accession numbers

The nucleotide sequences of *ampC* in seven isolates were submitted to GenBank and assigned accession numbers EU118260, EU118261, EU118262, EU118263, EU118264, EU118265, EU118266. The sequences of *ompA* from five isolates have also been submitted: EU332795, EU332796, EU332797, EU332798, and EU332799.

RESULTS

All isolates were characterized by ribotyping, rep-PCR, and PFGE (Fig. 1). They were confirmed as *A. baumannii* by ribotyping which reliably identifies isolates to the species level [12].

Isolates of ribotype 28-5

Nineteen isolates were assigned to a ribotype pattern (28-5) that encompasses many of the carbapenem-resistant isolates gathered during our surveillance studies [13]. All were resistant to ceftazidime, and 16 were resistant to meropenem. Based on rep-PCR and PFGE patterns, two major subtypes were recognized (groups α and β; Table 1). Group α consisted of eight isolates with similar rep-PCR and PFGE types. Six of these isolates had a distinctive Pro^{238}→Arg change in AmpC, and seven had an Ala insertion between amino acids 242 and 243 (Table 1). Consistent changes were found in OmpA (Gly^{52}→Ser), AdeR (Val^{120}→Ile and Ala^{136}→Val), and AbeM (Val^{143}→Ile) in the eight isolates. One isolate in this group had two additional changes in OmpA (GenBank no. EU332796), and had an *ompA* sequence that was identical to that of an isolate representative of the South East clone endemic to England [21, 22]. While all eight α isolates carried a mutation in *adeB* at nucleotide 1974, two had additional mutations at nucleotide 1902, and would have been excluded from this group according the typing scheme using this gene [11].

Ten isolates in ribotype 28-5 fell into a second distinct rep-PCR group and PFGE group, comprising the β group (Table 1). These 10 isolates shared changes involving OmpA and AdeR seen in the preceding α group. However, none of the isolates had the Val^{143}→Ile change in AbeM. Only one of the 10 isolates possessed the Pro^{238}→Arg amino-acid change and the Ala insertion seen in AmpC of the α group; this isolate may indeed be an intermediary between the α and β groups in this ribotype. All 10 isolates in the β group shared a single mutation in *adeB* at nucleotide 1974, as previously noted in the α group.

One isolate in ribotype 28-5 had a rep-PCR pattern and changes in OmpA and AdeR characteristic of the β group. However, this isolate had a unique PFGE pattern, and was therefore considered a unique strain. While this isolate possessed the mutation at nucleotide 1974 in *adeB* seen in the β group, it also harboured two other mutations, and would have been considered unique according the *adeB* typing scheme [11].

https://doi.org/10.1017/S0950268808000824 Published online by Cambridge University Press

Table 1. *Amino-acid changes characteristic of groups of clonally related isolates of* A. baumannii

	Rep-PCR group	PFGE group	Signature amino-acid changes (number of isolates with change/number of isolates in group) AmpC	OmpA	AdeR	AbeM
Isolates belonging to ribotype 28-5						
α Group ($n=8$)	A1, A2, A3	Ia, Ib, Ic, Id	$Pro^{238}\rightarrow Arg$ (6/8) Insert $^{243}Ala^{243}$ (7/8)	$Gly^{52}\rightarrow Ser$ (8/8)	$Val^{120}\rightarrow Ile$ (8/8) $Ala^{136}\rightarrow Val$ (8/8)	$Val^{143}\rightarrow Ile$ (8/8)
β Group ($n=10$)	B1, B2	IIa, IIb, IIc, IId	None	$Gly^{52}\rightarrow Ser$ (10/10)	$Val^{120}\rightarrow Ile$ (10/10) $Ala^{136}\rightarrow Val$ (10/10)	None
Isolates belonging to ribotype 35-2						
ζ Group ($n=8$)	C	IIIa, IIIb, IIIc, IIId	None	(8/8)*	$Leu^{142}\rightarrow Ile$ (8/8) $Val^{243}\rightarrow Ile$ (8/8)	$Val^{171}\rightarrow Ile$ (8/8)
η Group ($n=2$)	E1, E2	VI	(2/2)†	None	$Val^{14}\rightarrow Ile$ (2/2) $His^{195}\rightarrow Gln$ (2/2)	None
Isolates belonging to ribotype 66-3						
λ Group ($n=4$)	D1, D2	IVa, IVb	$Ala^{224}\rightarrow Asp$ (3/4) $Pro^{244}\rightarrow Ala$ (3/4) Insert $^{243}Leu^{244}$ (3/4)	(4/4)*	$Leu^{142}\rightarrow Ile$ (2/4)‡ $Val^{243}\rightarrow Ile$ (2/4)	$Val^{171}\rightarrow Ile$ (4/4)
Isolates belonging to ribotype 69-4						
ν Group ($n=4$)	U	Va, Vb	$Lys^{150}\rightarrow Gln$ (4/4) $Ser^{167}\rightarrow Pro$ (4/4) $Ser^{257}\rightarrow Lys$ (4/4) $Arg^{283}\rightarrow Phe$ (4/4)	$Ala^{89}\rightarrow Pro$ (4/4) $Ser^{90}\rightarrow Val$ (4/4) $Thr^{144}\rightarrow Asn$ (4/4)	$Val^{120}\rightarrow Ile$ (3/4) $Asp^{181}\rightarrow Glu$ (3/4)	$Ser^{227}\rightarrow Asn$ (4/4) $Gln^{387}\rightarrow Glu$ (4/4)

* All had mutations leading to the following changes: S38T;N48E;A89L;S90A;D133E;F134I;D135P;G136D;V137L;N138S;R139Y;G140H;T141N; deletion^{142}RGT^{144};S145D;A173G;A177F;E180K.

† Both had mutations leading to the following changes: G99A;D110N;S167P;F193S; only the first 209 amino acids could be determined.

‡ Two isolates had non-amplifiable *adeR*.

Isolates of ribotype 35-2

Twelve isolates were included in a second ribotype (35-2) that also represents a large percentage of carbapenem-resistant isolates in our city (Table 1). All were resistant to ceftazidime, and six were resistant to meropenem. Eight of these isolates were clearly related by rep-PCR and PFGE and comprised group ζ. These eight isolates also possessed an AmpC that closely resembled ADC-1, and had multiple changes in OmpA (17 amino-acid alterations and a deletion of three amino acids). The sequence of *ompA* from isolates in this group was identical to the *ompA-2* allele from a strain recovered from France in 2001 and characteristic of two widely disseminated European clones [21, 23]. The eight ζ isolates also had distinctive amino-acid changes in AdeR ($Leu^{142}\rightarrow Ile$ and $Val^{243}\rightarrow Ile$) and AbeM ($Val^{171}\rightarrow Ile$), and shared 22 mutations involving *adeB*.

Two identical isolates in ribotype 35-2 were unrelated to the preceding eight (ζ) isolates by both rep-PCR and PFGE. The *ampC* gene for these two isolates was non-amplifiable; using an internal primer (used for sequencing), the first 628 bp were able to be amplified, and revealed several unique changes (Table 1). These two isolates also had unique changes affecting AdeR and mutations involving *adeB*. The remaining two isolates in ribotype 35-2 were unique by rep-PCR and PFGE. One isolate had a premature stop codon in AmpC at amino acid 143 and had the signature changes in OmpA, AdeR, and AbeM of the α group. The remaining isolate had unique changes in the four genes.

Isolates of ribotype 66-3

Five isolates were classified as ribotype 66-3 (Table 1), the third largest ribotype among multidrug-resistant isolates. Four of these were resistant to ceftazidime and meropenem, and were related by rep-PCR and PFGE (group λ). Three λ isolates shared multiple distinctive mutations affecting AmpC (including

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Ala^{224}→Asp, Pro^{244}→Ala, and an insertion of a Leu) and OmpA; however, one of these three had unique mutations affecting *adeB*. The fourth isolate lacked the changes in AmpC seen in the other three isolates, but did possess the mutations affecting OmpA. Although the four group λ isolates had distinctive changes in AmpC and *adeB*, their changes in OmpA, AdeR, and AbeM were similar to those observed in the ζ group. The fifth isolate of ribotype 66-3 was unrelated by rep-PCR and PFGE, and had multiple unique changes affecting AmpC and OmpA.

Antimicrobial susceptible isolates

Seven isolates that were susceptible to piperacillin-tazobactam, ceftazidime, and carbapenems were selected for further analysis. Four isolates, gathered from patients at three different hospitals, shared similar ribotype (69-4), rep-PCR, and PFGE patterns (group ν; Table 1). These isolates also shared several unique mutations affecting AmpC, including the following amino-acid changes: Lys^{150}→Gln, Ser^{167}→Pro, Ser^{257}→Lys, and Arg^{283}→Phe. These four isolates had identical OmpA and AbeM, and three had identical AdeR alterations. There were 19 mutations in *adeB* that were characteristic of the four isolates in this group. The remaining three susceptible isolates each had unique ribotype, rep-PCR, and PFGE patterns. One isolate possessed similarities in OmpA compared to the λ and ζ groups, however, the changes in AmpC, AdeR, and AbeM were dissimilar. The remaining two unique isolates had distinct sequences involving these genes.

DISCUSSION

In prior surveillance studies involving the hospitals in Brooklyn, NY, it was documented that most of the multidrug-resistant *A. baumannii* isolates belonged to one of three ribotypes [13, 14]. In this report, further genotypic evaluation of isolates belonging to these three ribotypes was performed. It appears the multidrug-resistant *A. baumannii* isolates in our region fall into major groups with distinct predecessors. The isolates in one ribotype (28-5) possess similar mutations in *ompA*, *adeB*, and *adeR* (and overlapping mutations of *ampC*), but have two (α and β) subtypes with distinct rep-PCR and PFGE patterns and *abeM* sequences. While it is possible that ribotyping has less discriminatory power and these two subtypes are unrelated, it seems more likely that these two subtypes are derived from a common ancestor. Most isolates in the second (35-2) and third (66-3) ribotypes also had distinct rep-PCR, and PFGE patterns and *ampC* and *adeB* genes. However, these two share multiple changes in OmpA and unique mutations involving *abeM* and *adeR*. While these two types (ζ and λ) may be ancestrally related, it is also possible they represent unique strains that have acquired similar genetic changes.

Examination of a small number of β-lactam susceptible isolates did not reveal strains that were closely related to the multidrug-resistant isolates. None of the susceptible isolates appeared to be related to the α and β groups, and the susceptible strains generally possessed unique mutations affecting *ampC*, *ompA*, *adeB*, *adeR*, and *abeM*. When compared to the susceptible strains, it appears the multidrug-resistant isolates do not share the same genes associated with antimicrobial resistance.

Our results are analogous to the fingerprinting studies involving *A. baumannii* isolates originating from Europe, South Africa, and Israel [21, 24]. As with our New York City isolates, multidrug-resistant nosocomial isolates from Europe and South Africa tend to fall into one of three large ribotypes, and within each ribotype are distinct genotypes (by PFGE) [24]. Moreover, remarkable similarities in *ompA* are observed in isolates from New York City and Europe [21]. Reported clonal groups carrying allele I of *ompA* include the South East clone, European clone II (corresponding to European ribogroup II [24]), T strain, and Oxa-23-clone I found in Europe and Israel [21]. Isolates belonging to our α and β genotypes (that share a single ribotype) have similar *ompA* sequences as these isolates. Clonal groups carrying allele II of *ompA* include European clone I (corresponding to European ribogroup I [24]), Oxa-23-clone 2, and the French strain AYE-VEB-1 [22]. This allele was also recovered in a distinctive group that included European clone III [21]. Two distinctive genotypic groups from New York City (ζ and λ, with different ribotypes) also share allele II of *ompA*. Moreover, many of the New York City isolates share sequences of *adeB* that have been documented in isolates from Europe [11, 25]. Given these similarities, it appears the European and American isolates may indeed be ancestrally related. As a consequence of the worldwide distribution of multidrug-resistant *A. baumannii*, further development of MLST schemes [10, 21] will be essential to

determine if isolates from geographically diverse areas share genetic lineages.

ACKNOWLEDGEMENTS

This study was supported by the National Institutes of Health (RO1 AI070246-01A1).

DECLARATION OF INTEREST

None.

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