Antibiotic resistance determinants in nosocomial strains of multidrug-resistant Acinetobacter baumannii

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Received 28 May 2008; returned 23 June 2008; revised 7 October 2008; accepted 7 October 2008

Objectives: To investigate the presence of resistance genes in nosocomial multidrug-resistant (MDR) Acinetobacter baumannii isolated from outbreak and sporadic settings.

Methods: Thirty-two A. baumannii isolates were collected, 13 of which were involved in two outbreaks from different hospitals, which resulted in four deaths. The remaining 19 isolates were collected sporadically over 5 years from two other hospitals. The MICs of 25 antibiotics were determined for each isolate. PCR screening was carried out to identify possible genes that contributed to each resistance phenotype. Repetitive extragenic palindromic-PCR (REP-PCR) was performed to assess isolate clonality in conjunction with genotyping data.

Results: Between eight and 12 resistance determinants were detected in the 32 MDR A. baumannii isolates examined. These resistance determinants included the genes blaOXA-23 and ampC, with the upstream element ISaba1 promoting increased gene expression and subsequent resistance to carbapenems and cephalosporins, respectively. In all isolates, resistance to quinolones and fluoroquinolones was conferred by an S83L mutation in GyrA. Twenty-eight of the 32 isolates were also positive for tet(B), a tetracycline resistance determinant, blaTEM-1, which contributed to β-lactam resistance, and strB, which contributed to aminoglycoside resistance. Class 1 integrons that harboured aacC1, adaA1, qacEΔ1 and sul1 were identified in 10 of the 32 isolates (31%) together with the kanamycin resistance gene, aphA1. A putative trimethoprim resistance gene, folA, was also identified in all isolates. REP-PCR together with genotyping identified three main clonal types.

Conclusions: Isolates of A. baumannii from both outbreak and sporadic cases possess at least eight resistance gene determinants that give rise to the MDR phenotype.

Keywords: A. baumannii, OXA-23, multidrug resistance

Introduction

Acinetobacter spp. have been recognized over the last two decades as important opportunistic pathogens. Extensive use of antimicrobial chemotherapy in clinical environments has contributed to the emergence and dissemination of nosocomial Acinetobacter baumannii infections. These infections are difficult to treat due to the organism’s multidrug-resistant (MDR) phenotype, which includes resistance to β-lactams, aminoglycosides, fluoroquinolones and more recently, carbapenems. A. baumannii in particular is capable of causing a wide spectrum of disease including urinary tract infections, pneumonia and meningitis, especially in intensive care units (ICUs), and is associated with high mortality rates. Lately, several clinical outbreaks associated with carbapenem-resistant A. baumannii have been described1,2 and alternative treatment options are limited.

Currently, there is a relative paucity of data on the number and type of resistance genes and mechanisms that may be present in MDR A. baumannii strains. Many studies focus only on a single resistance phenotype and its associated resistance gene. In the current study, four discrete cohorts of A. baumannii were investigated. As all these isolates were MDR, we aimed to identify the gene/s responsible for resistance to 25 antibiotics from a range of classes. This is the first study that attempts to determine the resistance gene profile of A. baumannii responsible for nosocomial ICU outbreaks and sporadic infections.

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Materials and methods

Bacterial isolates and plasmids

A total of 32 isolates of *A. baumannii* were collected from four different hospitals in Sydney, Australia. *A. baumannii* was identified by colonial appearance on MacConkey agar, Gram staining, antibiotic susceptibility testing using disc diffusion, growth in oxidative fermentative media, resistance to ampicillin and the presence of *bla*OXA-51-like. Five isolates (termed cohort A) of *A. baumannii* (A91, A93, A94, A96 and A97) were collected from five patients during an ICU outbreak that resulted in two deaths in May 2005. The second ICU outbreak cohort, termed cohort B, consisted of eight MDR *A. baumannii* (B1–B8) and was obtained from another large Sydney hospital in March 2007. The bacterial infection in three of these latter cases was septicemia, as a result of which two patients died. Sporadic isolates were defined as strains that were not associated with an outbreak, and the remaining two cohorts fell into this category. Cohort C, obtained from the standard wards of a third hospital, consisted of 10 different isolates (C2–C5, C8, C13–C15, C18 and C20) originating from wound, catheter, blood and urine samples, as well as environmental surfaces including bed railings and air-conditioning vents (isolates C3 and C4). Eight of the cohort C isolates were collected randomly between September 2006 and February 2007, and the remaining two isolates (C18 and C20) were isolated in November 2002. Cohort D consisted of nine MDR *A. baumannii* isolates (D1–D3, D5–D9 and D12) collected sporadically between June 2006 and May 2007. These isolates (none of which were sourced from the ICU) were collected from catheters, wounds and urine samples. A single *A. baumannii* isolate named A95, which was susceptible to many antibiotics, was isolated from the same hospital as cohort A and was used as a control in all procedures. Another control isolate, *Acinetobacter calcoaceticus* (UNSW 031600), was obtained from the University of New South Wales Microbiology Culture Collection (http://www.babs.unsw.edu.au/about/centres/micro_culture.html). *Escherichia coli* strain ACM 5185 was used as a control for MIC determination.

Quantitative antibiotic susceptibility testing

MIC determination of each antibiotic was performed using an agar dilution technique. Susceptibilities of *A. baumannii* to 25 different antibiotics were tested as shown in the Results section. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Detection of resistance determinants

Integron detection, characterization and gene cassette amplification were carried out using previously described PCR methods. Primers used to detect other non-integron-associated resistance genes are shown in Table 1. Primers for the detection of tetracycline resistance determinants, the *β*-lactamase gene *bla*OXA-51-like and *ISAba1* were used as previously described. Sequencing and DNA analysis of all PCR products were carried out using methods described elsewhere. To identify mutations associated with quinolone and fluoroquinolone resistance, the 344 bp quinolone resistance determining region (QRDR) of the DNA gyrase gene, *gyrA*, was amplified and restricted using the method of Vila et al., with the primers Vila 95 gyrA-F12 and PW40 (Table 1). Sequencing was also used to confirm the presence of a mutation in *gyrA*.

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<sup>a</sup> All primers described in this table were designed in this study.
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AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; TIM, ticarcillin/clavulanate; TZP, piperacillin/tazobactam; AMC, amoxicillin/clavulanate; MEM, meropenem; IPM, imipenem; TIG, tigecycline; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; STR, streptomycin; SPT, spectinomycin; TET, tetracycline; TMP, trimethoprim; PMX, polymyxin B.

*The MICs of the following antibiotics were identical for all 32 isolates: cefazolin, ≥128 mg/L; cefalexin, ≥128 mg/L; chloramphenicol, ≥64 mg/L; ciprofloxacin ≥64 mg/L; nalidixic acid, ≥256 mg/L; and sulfafurazole, ≥128 mg/L.*
DNA extraction and repetitive extragenic palindromic-PCR (REP-PCR) for clonality assessment

Genomic DNA extractions were carried out using the Bio-Rad AquaPure Genomic DNA Extraction Kit (Bio-Rad) according to the manufacturer’s instructions. REP-PCR was performed using the method and primers of Bou et al.13

Trimethoprim resistance determination

In order to identify the trimethoprim resistance gene, genomic DNA was extracted from a representative isolate, A. baumannii A91, and then restricted with EcoRI (Promega, WI, USA). The restricted fragments were then ligated with EcoRI cut pUC18. Resultant transformants were then patched onto LB agar containing 25 mg/L trimethoprim. Plasmids conferring trimethoprim resistance were extracted using the Bio-Rad Quantum Plasmid Miniprep Kit (Bio-Rad) and sequenced using vector primers14 and those listed in Table 1. Shotgun cloning led to the isolation of the putative trimethoprim resistance gene, folA, which was then amplified from the genomic DNA of A91 using primers PW221 and PW222 (Table 1), cloned into pTrcHis2A and transformed into E. coli BL21. Subsequent transformants were selected on trimethoprim and subjected to MIC determination. Phylogenetic analysis was carried out as previously described.11

Results

Antibiotic susceptibility testing

All 32 isolates of MDR A. baumannii were tested for antibiotic susceptibility by determining MICs of a range of 25 antibiotics (Table 2). The most resistant isolates (C3, C5, C8, C15, C18, C20, D8, D9 and D12) were resistant to 23 of the 25 antibiotics tested (Table 2). All 32 isolates showed high levels of resistance to cefalexin, cefazolin, chloramphenicol, sulfafurazole and nalidixic acid (Table 2). Twenty-eight isolates demonstrated an additionally high level of resistance to ampicillin, ticarcillin/clavulanate, amoxicillin/clavulanate, streptomycin and spectinomycin (Table 2). The majority of isolates were also resistant to tigecycline, with only four isolates (C4, C13, C14 and D1) and the control isolate A95 susceptible to the drug. Of the 25 antimicrobials tested, the most effective was polymyxin B, followed by amikacin and tobramycin (Table 2).

Detection and characterization of integrons

In order to assess the presence of integrons in A. baumannii, PCR screening for intI genes was performed. Class 1 integrons were found in 10 of the 32 isolates (31%); including the five isolates from cohort A, a single positive isolate from cohort C (C20) and four of nine isolates from cohort D (D2, D3, D6 and D7) (Table 3). No integron positive isolates were found in the outbreaks and no class 2 or 3 integrons were found within the cohorts. Subsequent PCR amplification and sequencing of the cassette arrays revealed that all 10 integron-positive A. baumannii isolates contained a 2.6 kb gene cassette array containing aacC1, orfX, orfX’ and aadA1, which conferred resistance to gentamicin, streptomycin and spectinomycin.
Multiresistance in *A. baumannii*

**Streptomycin resistance**

The *aadA1* gene cassette was present in 10 integron-positive isolates, yet all 32 isolates were resistant to streptomycin. Thus, other determinants conferring resistance to this antibiotic were sought. The presence of two genes, *strA* and *strB*, recently implicated in streptomycin resistance in *A. baumannii*, was assessed by PCR. Twenty-eight of the 32 isolates contained the single *strB* gene (Table 3), while the *strA* gene was not found in any isolate. The control isolate A95 was fully susceptible to both spectinomycin and streptomycin and lacked *aadA1*, *strA* and *strB* resistance genes.

**Kanamycin resistance**

Another aminoglycoside resistance gene, *aphA1*, encodes kanamycin resistance in *A. baumannii*. The *aphA1* resistance gene was identified in 10 of the 22 kanamycin resistant isolates (A91, A93, A94, A96, A97, C20, D2, D3, D6 and D7), the same 10 isolates that harboured the class 1 integron (Table 3). These 10 isolates were highly resistant to kanamycin (MIC > 512 mg/L) (Table 2).

**Genetic basis of β-lactam resistance**

Resistance to β-lactams was observed in all isolates. The intrinsic *ampC* β-lactamase gene was identified in all 32 isolates (Table 3). The presence of an insertion element, ISAba1, immediately upstream of *ampC* has been attributed to increased *ampC* expression resulting in an extended hydrolysis profile and high-level cephalosporin resistance.17,18 This insertion element was found upstream of the *ampC* gene in 28 of 32 isolates (88%) (Table 3). These 28 isolates were resistant to a range of cephalosporins, including cephalosporin (Table 2). The four isolates that lacked the insertion sequence (D2, D3, D6 and D7) had reduced MICs of cephalosporin, cefotaxime, cefoxitin and ticarcillin/clavulanate but were still highly resistant to cefalexin and cefazolin (Table 2).

The gene *bla*OXA-23 was detected in 28 of the 32 isolates (88%) (Table 3). The product, TEM-1, hydrolyses penicillin and ampicillin, but does not confer resistance to β-lactamase inhibitors.19 The same four isolates (D2, D43, D6 and D7) that lacked ISAba1 upstream of *ampC* also lacked the *blaTEM-1* gene (Table 3).

**Carbapenem resistance is conferred by *bla*OXA-23 with an upstream ISAba1**

In *A. baumannii*, carbapenem-hydrolysing β-lactamases of Ambler class B and D play a significant role in providing resistance to carbapenems.20–22 Two oxacillinases were identified in this study, *bla*OXA-23 and *bla*OXA-51-like. The gene *bla*OXA-23 was amplified from 28 isolates, all of which harboured an upstream insertion element, ISAba1 (Table 3). This results in an increase in carbapenemase activity and leads to imipenem and meropenem resistance, which was reflected in the MDR phenotype (Table 2). The four susceptible isolates from cohort D (D2, D3, D6 and D7) lacked both the gene and the insertion sequence (Table 2) and were susceptible to the carbapenems (Table 2). The intrinsic β-lactamase gene *bla*OXA-51-like was amplified in all 32 *A. baumannii* isolates (Table 3). However, *bla*OXA-51-like confers insufficient carbapenemase activity for resistance without the presence of an upstream insertion element.23 No upstream ISAba1 was identified through PCR screening; therefore, it is unlikely that the *bla*OXA-51-like gene was involved in carbapenem resistance.

**Quinolone and fluoroquinolone resistance is conferred by an S83L gyrA mutation**

All 32 isolates studied were resistant to nalidixic acid and ciprofloxacin. RFLP analysis and sequencing revealed all the isolates of *A. baumannii* contained a nucleotide mutation in the QRDR of gyrA (Table 3). The mutation results in an amino acid change from S83L and is a well-recognized resistance mutation.12 The control, A95, which did not contain this mutation, was susceptible to the quinolones.

**Tetracycline resistance**

In clinical isolates of *A. baumannii*, tet(A) and tet(B) confer tetracycline resistance; however, tet(B) is more prevalent.8 All 32 isolates were resistant to tetracycline, and tet(B) was identified by PCR in 28 isolates (Table 3). The remaining four isolates (D2, D3, D6 and D7) did not contain tet(A) or tet(B) genes (Table 3); however, they were still resistant, albeit with lower MIC values of tetracycline (16 mg/L), when compared with the rest of the isolates (MIC >256 mg/L) (Table 2). The control isolate, A95, was susceptible to tetracycline and also did not contain tet(A) and tet(B).

**folA is a putative trimethoprim resistance determinant in MDR *A. baumannii***

A putative trimethoprim resistance gene was found by shotgun cloning a representative isolate, A91. Selection of clones on agar plates supplemented with trimethoprim resulted in the isolation of a resistant plasmid, named pITN84. Sequencing 4.5 kb of the 16 kb insert revealed the presence of a 510 bp ORF, *folA*, which encoded a dihydrofolate reductase (Dfr), an enzyme that can confer trimethoprim resistance. This gene was present in all 32 trimethoprim-resistant *A. baumannii* isolates (Table 3). The 510 bp *folA* gene from one isolate, A91, was amplified and cloned into pTrcHis2A. Expression of the enzyme in *E. coli* strain BL21 resulted in high-level trimethoprim resistance, with a MIC >1024 mg/L, while the *E. coli* host strain showed an MIC of ≤0.5 mg/L.

The 169 residue *folA* product demonstrated 99.4% identity with the Dfr found in *A. baumannii* ACICU,24 and 98.8% identity with the Dfrs in *A. baumannii* ATCC 17978, *A. baumannii* AYE and *A. baumannii* SDF25 (Figure 1). The Dfr in this study was also related to other *folA*-encoded Dfrs, including those from *Acinetobacter* sp. ADP1 (72%, 122/169 amino acids), *Acinetobacter* sp. ADP1 (43%, 81/188 amino acids), *Acinetobacter* sp. ADP1 (72%, 122/169 amino acids) and *Acinetobacter* sp. ADP1 (35.9%, 66/184 amino acids) and *DfrA16* (35.7%, 61/171 amino acids) (Figure 1).

**REP-PCR and clonality of isolates**

In order to determine the clonality of the 32 *A. baumannii* isolates, REP-PCR was carried out on genomic DNA. Two REP-PCR patterns or ‘genomic fingerprints’ were observed (see Figure S1, available as Supplementary data at JAC Online
Multiresistance in A. baumannii

Discussion

A. baumannii causes a significant number of nosocomial outbreaks worldwide, which commonly occur in settings with high antibiotic selective pressures, such as ICUs. Therapeutic options for the treatment of MDR A. baumannii are becoming increasingly limited. All the isolates of A. baumannii from the current study exhibited an MDR phenotype, with the nine most resistant isolates susceptible to only two of the 25 antibiotics tested. There were no significant differences in the genotypic and phenotypic profiles, as the 19 sporadic and environmental surface (C3 and C4) A. baumannii isolates possessed the same resistance genes as the 13 outbreak isolates.

Insertion sequences containing promoters that alter the levels of gene expression have been documented in various Gram-negative bacteria, including Acinetobacters.12,17 Carbapenem resistance in A. baumannii is often mediated through increased oxacillinase gene expression, which is driven by the promoter region of an upstream ISAba1.23 In this study, ISAba1 was found upstream of both ampC and blaOXA-23, thus giving rise to cephalosporin and carbapenem resistance. The finding of ISAba1 upstream of two different genes in the same bacteria suggests that under a selective pressure it is able to transpose, providing a strong promoter sequence for multiple resistance genes. The blaOXA-23 gene has previously been isolated in conjunction with the same integron described in the present study from A. baumannii outbreak strains from pan-Europe,27 Greece28 and previously from Australia.2 The integron array found in this study, together with the gene blaOXA-23, has also been described in an A. baumannii outbreak clone in the UK, designated OXA-23 clone 2.29 This clone, first isolated in July 2003, continues to be isolated in hospitals around the UK.30 Therefore, the OXA-23 clone 2 may also contain the other resistance determinants such as ISAba1/ampC, blataTEM-1, tet(B), strB, folA and the gyrA mutation as the isolates found in this study.

In A. baumannii strains AYE and ACICU, aphA1 was located between two IS26 elements just upstream of a class 1 integron on large ‘resistance islands’.15,25 Isolates in this study containing an integron also possessed the kanamycin resistance gene aphA1, and the co-occurrence of these two elements has been shown previously.27 Therefore, we hypothesize that the aphA1 gene and the integron are associated with one another.

A gene conferring putative trimethoprim resistance was found by shotgun cloning. This gene was present in all 32 trimethoprim-resistant isolates and differed in only one nucleotide from folA genes from four clinical isolates of A. baumannii.24–26

While trimethoprim resistance seems to be intrinsic in clinical A. baumannii isolates, no gene has yet been described that mediates this resistance. The role of FolA and other housekeeping Dfrs in conferring trimethoprim resistance has previously been reported.31,32 Overexpression of chromosomal Dfrs is also a recognized mechanism of trimethoprim resistance.33,34 When overexpressed in E. coli, the folA gene identified in this study confers high-level trimethoprim resistance; however, more evidence is needed to confirm its ability to confer trimethoprim resistance in its natural context.

It has been shown in this study that MDR A. baumannii can possess at least eight resistance determinants that give rise to its MDR phenotype. Although resistance was observed against amikacin, tobramycin, chloramphenicol and tigecycline, a genetic basis for the resistance was not accounted for. In addition, there were a number of isolates in which a resistance phenotype was observed, but no specific resistance determinant was identified. This is an indication that there are as yet unidentified resistance genes present in these cohorts of MDR A. baumannii.

The resistance determinants MDR A. baumannii possess, such as an integron, the β-lactam resistance genes blaOXA-23, ampC and blataTEM-1, aminoglycoside, tetracycline and trimethoprim resistance genes, and a gyrA mutation conferring quinolone resistance, contribute to the MDR profile of nosocomial strains within hospitals in Australia. These resistance determinants were present in outbreak as well as sporadic isolates, which emphasizes the multitude of resistance genes A. baumannii is capable of possessing.

Acknowledgements

This work was presented in part at the One hundred and eighth Annual General Meeting of the American Society for Microbiology, Boston, MA, 2008 (Abstract A-042). We thank Professor Bill Rawlinson, Dr Christopher McIver for his assistance in strain identification, Dr Alexander Outhred for his assistance with clinical data, Dr John Merlino and Dr Tom Gottlieb, Dr Peter Taylor and Kerry Varettas and Dr Clarence Fernandez for strain provision and Aileen Oon for her assistance with MIC determination.

Funding

No specific funding was received for this study. J. K. M. was supported by a University of New South Wales postgraduate award.
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