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Rapid detection of antibiotic resistance in *Acinetobacter baumannii* using quantitative real-time PCR

Reyes Martín-Peña, Juan Domínguez-Herrera, Jerónimo Pachón and Michael J. McConnell*

Unit of Infectious Diseases, Microbiology, and Preventive Medicine and Biomedical Institute of Seville (IBiS), University Hospital Virgen del Rocío/CSIC/University of Sevilla, 41013 Sevilla, Spain

*Corresponding author. Tel: +34-955923104; E-mail: mcconnell.mike75@gmail.com

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Objectives: The rapid detection of antibiotic resistance in clinical isolates of *Acinetobacter baumannii* would shorten the period during which patients receive empirical therapy and facilitate the early initiation of directed antibiotic therapy. The objective of this study was to evaluate the ability of a real-time PCR assay to detect antibiotic resistance to four clinically relevant antibiotics from different antibiotic classes in clinical isolates of *A. baumannii*.

Methods: The growth of 48 clinical isolates of *A. baumannii* with a broad range of MICs of imipenem, ciprofloxacin, colistin and amikacin was evaluated using a real-time PCR assay targeting a highly conserved region of the *ompA* gene. Fold changes in the number of copies of genomic DNA after 6 h of growth were used to determine resistance and the results were compared with those obtained using broth microdilution.

Results: The results obtained using the real-time PCR assay were concordant with broth microdilution for 184 of 192 determinations (95.8%). The global values for specificity (97.5%), sensitivity (92.9%), positive predictive value (95.6%) and negative predictive value (96.0%) indicated that the real-time PCR assay was able to reliably differentiate between resistant and non-resistant strains.

Conclusions: The use of real-time PCR to monitor bacterial growth in the presence of antibiotics is effective for rapidly identifying antibiotic resistance in *A. baumannii*.

Keywords: susceptibility testing, multidrug resistance, empirical therapy

Introduction

Acinetobacter baumannii can produce different types of infections, most typically nosocomial pneumonia and bloodstream infections in critically ill patients.[1] These infections are associated with high mortality[2–4] and the global emergence of strains with resistance to antibiotics from multiple classes (i.e. multidrug-resistant and pan-drug-resistant strains) has reduced the number of drugs that retain activity against this pathogen. With certain types of infections produced by *A. baumannii*, e.g. ventilator-associated pneumonia, empirical therapy is administered until a microbiological diagnosis identifying the causative agent of infection and its antibiotic resistance profile is obtained. Once this information is known, directed therapy with known activity against the causative organism can be initiated. The application of rapid diagnostic tests to the microbiological diagnosis of *A. baumannii* infections could therefore improve patient outcomes by reducing the time that elapses before the patient receives directed therapy. In the present study, we evaluate the use of a real-time PCR assay to rapidly detect resistance to imipenem, ciprofloxacin, colistin and amikacin in clinical isolates of *A. baumannii*.

Materials and methods

Bacterial strains and MIC determination

Forty-eight *A. baumannii* clinical isolates were used for this study. Thirteen of the isolates (9 resistant to colistin) were collected during an outbreak of colistin-resistant *A. baumannii* in the Hospital Virgen del Rocío in 2002;[5] the remaining 35 clinical isolates were determined to be clonally distinct by repetitive extragenic palindromic sequence-based PCR (REP-PCR).[6] Unless otherwise stated, the strains were maintained by growth on blood agar plates and in cation-adjusted Mueller–Hinton broth (MHB). The MICs of imipenem, ciprofloxacin, colistin and amikacin were determined by broth microdilution and resistance was defined using the breakpoints defined by the CLSI.[7]

Bacterial growth conditions and DNA isolation

A. baumannii colony material from blood agar plates was resuspended in physiological saline and the OD_{600} adjusted to 0.6, which corresponds to

$\sim 1\times10^8$ cfu. The suspension was diluted 1:100 in 2× MHB and 100 μL of this bacterial suspension was added to the well of a 96-well plate containing 100 μL of the antibiotic solution at the appropriate concentration. The final antibiotic concentration in each well after addition of the bacterial suspension was 2-fold less than the breakpoint concentration for resistance as defined by the CLSI (final concentrations: 8 mg/L for imipenem, 2 mg/L for ciprofloxacin, 2 mg/L for colistin and 32 mg/L for amikacin). For 0 h timepoint samples, 150 μL of the bacteria/antibiotic mixture was collected immediately after addition of the bacterial suspension and the bacteria were pelleted by centrifugation at 5000 **g** for 10 min. Plates were incubated at 37°C for 6 h to allow for bacterial growth. After incubation, 150 μL of the bacteria/antibiotic mixture was collected and the bacteria were pelleted by centrifugation at 5000 **g** for 10 min. The bacterial genomic DNA was extracted from bacterial pellets obtained at 0 and 6 h for each strain using the Qiagen QIAmp DNA Mini Kit and eluted in a volume of 200 μL. All bacterial strains were tested in duplicate for each antibiotic.

Real-time PCR assay

The real-time PCR assay used for quantifying bacterial growth employs TaqMan chemistry for amplification of nucleotides 774–859 of the *A. baumannii ompA* gene (accession number: AY485227). The primers OmpA Forward (5′-TCTTGGTGGTCACTTGAAGC-3′) and OmpA Reverse (5′-ACTCTTGTGGTTGTGGAGCA-3′) and the probe 5′-AAGTTGCTCCAGT TGAACCAACTCCA-3′, 5′-labelled with 6-carboxyfluorescein and 3′-labelled with 6-carboxytetramethylrhodamine, were used.[8] A quantification standard consisting of the *ompA* gene cloned into the pGEM-T Easy vector was used.[8] Real-time PCR mixtures consisted of 10 μL of DNA (sample or quantification standard), 25 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), OmpA Forward and OmpA Reverse primers at a concentration of 300 nM each and the probe at a concentration of 100 nM in a total volume of 50 μL. The PCR parameters were 50°C for 2 min, 95°C for 10 min and then 38 cycles at 95°C for 30 s and 62°C for 1 min. All assays were performed on a Stratagene Mx3005P. In all assays, three concentrations of the quantification standard were assayed in duplicate (6.2×10^6, 6.2×10^3 and 62 copies/reaction) to determine the number of genome copies present in unknown samples.

Determination of resistance by real-time PCR

The fold increase in the number of bacterial genome copies during incubation in the presence of antibiotics was determined by dividing the number of genome copies present in samples collected after 6 h by the number of genome copies present at the 0 h timepoint. Based on a pilot study using a small number of strains in which real-time PCR results were compared with broth microdilution results, resistance using the real-time PCR assay was defined as a 4-fold increase in genome copies for imipenem, ciprofloxacin and colistin and a 3-fold increase for amikacin, during 6 h of incubation in the presence of the antibiotic.

Results

Determination of MICs by broth microdilution

As shown in Figure 1(a), the strains selected for this study had a broad range of MICs of the four antibiotics tested. The collection of strains also included representative resistant and non-resistant (susceptible or intermediate) strains to each antibiotic, with 18 resistant and 30 non-resistant to imipenem, 31 resistant and 17 non-resistant to ciprofloxacin, 9 resistant and 39 non-resistant to colistin and 18 resistant and 30 non-resistant to amikacin.

(a)

MIC mg/L	<0.03	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	>16
Imipenem				9	8	3	6	3	1			18
Ciprofloxacin			1	9	3	2	1	1		1	1	29
Colistin	10		16	10	2	1						9

MIC mg/L	0.25	0.5	1	2	4	8	16	32	64	128	256	>256
Amikacin			4	3	10	1	5	7	2	6	8	2

(b)

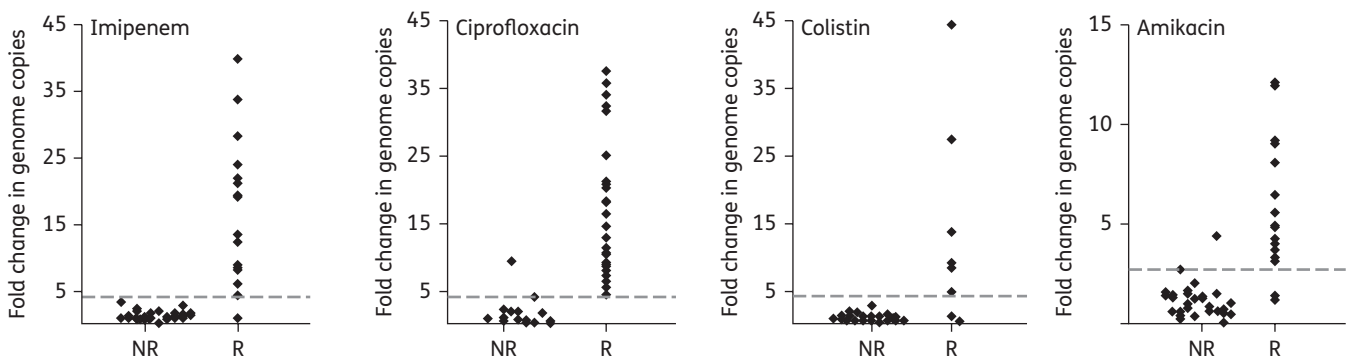

Figure 1. (a) MICs of imipenem, ciprofloxacin, colistin and amikacin for the 48 clinical isolates of *A. baumannii* used in the study. Values in each column represent the number of strains with the indicated MIC of each antibiotic. (b) The graphs represent the fold change in genomic DNA between the 0 and 6 h samples for imipenem, ciprofloxacin, colistin and amikacin for strains that were resistant (R) and non-resistant (NR) by broth microdilution. The broken line represents the cut-off used for classifying a strain as resistant or non-resistant by real-time PCR: 4-fold change for imipenem, ciprofloxacin and colistin and a 3-fold change for amikacin.

Table 1. Real-time PCR assay characteristics compared with broth microdilution for determination of antibiotic resistance in *A. baumannii*

Antibiotic	Agreement (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Imipenem	97.9	94.4	100	100	96.8
Ciprofloxacin	95.8	100	91.3	92.6	100
Colistin	95.8	77.8	100	100	95.1
Amikacin	93.8	88.9	96.7	94.1	93.6
Global	95.8	92.9	97.5	95.6	96.0

Determination of resistance by real-time PCR

In preliminary studies, three resistant and three non-resistant *A. baumannii* strains were grown in the presence of antibiotics for 2, 4, 6, 8 and 24 h before performing the real-time PCR in order to determine the minimum incubation time that allowed for differentiation between resistant and non-resistant strains. These studies indicated that an incubation time of 6 h was optimal (data not shown). The fold changes in genome copies after 6 h of growth of the 48 *A. baumannii* strains in the presence of the four antibiotics used in this study are shown in Figure 1(b). In the case of imipenem, the real-time PCR data were in agreement with broth microdilution for all strains, except for one strain (MIC <16 mg/L) that was identified as resistant by broth microdilution but did not demonstrate a 4-fold increase in genome copies. For ciprofloxacin, all strains were in agreement with broth microdilution data, except for two strains identified as non-resistant by broth microdilution (MICs 0.5 and 1 mg/L) that demonstrated a >4-fold increase in genome copies. In the case of colistin, two strains identified as resistant by broth microdilution (MICs <16 mg/L) did not reach the cut-off value of a 4-fold increase in genome copies to be classified as resistant by real-time PCR. For amikacin, one strain identified as non-resistant by broth microdilution (MIC 32 mg/L) showed a 4.4-fold increase in genome copies and two strains (MICs 64 mg/L) identified as resistant by broth microdilution did not reach the threshold of a 3-fold increase in genome copies.

Correlation of real-time PCR with broth microdilution

The results of the real-time PCR assay were compared with the results obtained using broth microdilution in order to calculate the sensitivity, specificity, positive predictive value and negative predictive value of the assay for each antibiotic and globally (Table 1). In general there was high overall agreement between the two assays (95.8% for all four antibiotics). However, it is of note that while the real-time PCR assay showed high sensitivity for detecting strains with resistance to imipenem and ciprofloxacin (94.4% and 100%, respectively), the sensitivity for detecting resistance to colistin and amikacin was somewhat lower (77.8% and 88.9%, respectively) due to a lack of increase in genome copies during the 6 h incubation time observed with a small number of strains characterized as resistant by broth microdilution. Possibilities that could explain the decreased sensitivity in the cases of colistin and amikacin are the presence of a lag phase before the initiation of exponential growth or heteroresistant strains (especially in the case of colistin) that require a longer growth time in order for resistant subpopulations to be detected.

Discussion

The use of rapid diagnostic tests with clinical isolates has the potential to reduce the time necessary for performing microbiological studies, therefore shortening the period during which empirical therapy is administered. This is especially important since a delay in the initiation of appropriate antibiotic therapy has been associated with higher patient mortality.[9,10] In the present study, the use of a real-time PCR for detecting resistance to four clinically relevant antibiotics in *A. baumannii* clinical isolates gave results that were highly concordant with the results obtained by broth microdilution. A recently published study using real-time PCR to measure bacterial growth in the presence of antibiotics reported similar results, with the agreement rate ranging from 87.1% to 100% depending on the antibiotic tested.[11] That study, however, did not evaluate the method with *A. baumannii* and did not test imipenem, colistin and amikacin. Two potential limitations of the method described here are that polymorphisms in the target sequences may affect the performance of the assay and that the assay provides only qualitative data (resistant or non-resistant). However, a major advantage of the technique described here is that, because the real-time PCR is measuring bacterial growth, this technique can in principle be used for detecting resistance to any antimicrobial, regardless of the resistance mechanism used by the bacterial strain. This is in contrast to a number of recently reported tests that aim to identify antibiotic resistance in *A. baumannii* through the amplification of genes that encode discrete resistance mechanisms (e.g. β-lactamases).[12–15] Potential limitations of these gene amplification-based approaches are that the presence of an antibiotic resistance gene may not correlate directly with phenotypic resistance and that these tests are unable to detect resistance due to novel resistance mechanisms, limitations that would probably not apply to the technique presented here.

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Transparency declarations

J. P. and M. J. M. own stock in and act as scientific advisors for Vaxdyn, S.L., a biotechnology company developing vaccines for multidrug-resistant bacteria, including *A. baumannii*. R. M.-P. and J. D.-H.: none to declare.

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