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Original Article

Antibiotic resistance patterns of *Acinetobacter calcoaceticus–A. baumannii* complex species from Colombian hospitals

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ABSTRACT

Introduction: Only automated phenotypic methods are currently used in Colombian hospitals for identifying isolates of the *Acinetobacter calcoaceticus–A. baumannii* complex (ACB). The phenotypical similarities in these species mean that they cannot be differentiated by manual or automated methods, thereby leading to their identification as *A. baumannii*, or ACB complex in clinical settings. Our objective was to identify to the species level 60 isolates, from four hospitals, evaluate their antibiotic susceptibility, and detect resistance-related genes.
Methods: 16S–23S rRNA internal transcribed spacer (ITS) region and *rpo*B gene partial sequences were amplified. Resistance genes for cephalosporin, carbapenem and aminoglycoside were detected by PCR. Possible mutations in the quinolone resistance-determining region (QRDR) were evaluated. The association of IS*Aba-1* with bla_{OXA} and bla_{ADC} genes was determined by PCR. Amplification products of ITS region, *rpoB* gene and some resistance genes were sequenced and compared using the BLAST tool.
Results: 16S–23S rRNA ITS region and partial *rpoB* gene sequence analysis allowed 51isolates to be identified as *A. baumannii*, 8 as *A. nosocomialis*, and 1 isolate as *A. pitti*. *A. baumannii* isolates were highly resistant to all antibiotics tested, while the others were susceptible to ciprofloxacin and ampicillin/sulbactam. Quinolone resistance, found only in *A. baumannii*, was associated with mutations in the QRDR region of *gyrA* and *parC* genes.
Conclusion: This is the first investigation in Colombia that has identified ACB complex species using molecular methods, and determined differences in antibiotic resistance and resistance genes among the species. It is of the highest importance to identify isolates to the species level for future resistance and epidemiology studies in our region.

Patrones de resistencia a antibióticos de especies del complejo *Acinetobacter calcoaceticus–A. baumannii* de hospitales en Colombia

Palabras clave:
Infecciones por *Acinetobacter*
Acinetobacter baumannii
ADN intergénico
Genes MDR

RESUMEN

Introducción: Actualmente, los hospitales en Colombia utilizan únicamente métodos fenotípicos automatizados para la identificación de aislamientos del complejo *Acinetobacter calcoaceticus – baumannii* (ACB). La similitud entre estas especies no permite que se diferencien por métodos fenotípicos ya sean estos manuales o automatizados, llevando a que los aislamientos se identifiquen como *A. baumannii* o como pertenecientes al complejo ACB en las instituciones hospitalarias. Nuestro objetivo fue identificar a nivel de especie, 60 aislamientos de cuatro hospitales, identificados como del complejo ACB, evaluar su resistencia a antibióticos y detectar genes de resistencia.
Métodos: Para la identificación de especies se amplificaron la región intergénica espaciadora de los genes 16S y 23S rRNA y la secuencia parcial del gen *rpo*B. Estos amplificados y algunos genes de resistencia se secuenciaron y se compararon utilizando la herramienta BLAST. Se detectaron por PCR genes de resistencia a cefalosporinas, carbapenemes y aminoglicósidos. Se evaluaron posibles mutaciones en la región determinante de resistencia a quinolonas (QRDR). Se determinó por PCR la asociación de IS*Aba-1*con los genes bla_{OXA} y bla_{ADC}.

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Resultados: Con las secuencias de la región ITS 16S-23S rRNA y el gen *rpo*B, se identificaron 51 aislamientos como *A. baumannii*, 8 como *A. nosocomialis* y 1 como *A. pittii*. *A. baumannii* fue altamente resistente a todos los antibióticos ensayados. Las otras dos especies fueron susceptibles a ciprofloxacina y ampicilina/sulbactam. La resistencia a quinolonas se detectó únicamente en *A. baumannii* y se asoció con mutaciones en la región QRDR de los genes *gyr*A y *par*C.
Conclusiones: Esta es la primera investigación en Colombia que identificó especies del complejo ACB usando métodos moleculares y determinó diferencias en la resistencia a antibióticos y en los genes de resistencia entre las especies. La identificación de los aislamientos a nivel de especie es de importancia para futuros estudios de resistencia y epidemiología en nuestra región.

Introduction

Acinetobacter calcoaceticus–A. baumannii complex comprises four genomic species: *A. calcoaceticus*, *A. baumannii*, *A. nosocomialis* (formerly genomic species 13TU[1]) and *A. pittii* (formerly genomic species 3[1]). Except for *A. calcoaceticus*, the other three species represent an important health threat as they are emerging as multidrug-resistant pathogens.[2]

As all these species are very close, genetically related, and difficult to identify by phenotypical methods using routine laboratory methods, it has been generally accepted to refer to them as a group (*ACB* complex),[2] although differences among them regarding their antibiotic resistance, epidemiology and pathogenicity have been also well documented.[3–5] Molecular methods as 16S–23S rRNA internal transcribed spacer (ITS) region analysis and *rpoB* gene fragment sequence analysis have been adopted for their identification.[6]

Regarding the antibiotic resistance, results from the SENTRY Antimicrobial Surveillance Program for Latin America and Brazil 1997–2001 indicate that resistant Gram-negative bacteria are much prevalent in Latin America than in North America and Europe. One of the main antimicrobial resistance problems is given by MDR (multidrug resistant) non-fermentative Gram-negative bacteria (*Acinetobacter* spp. and *Pseudomonas aeruginosa*).[7] This problem is also referred by Casellas,[8] indicating that species conforming *ACB* complex are resistant to a wide variety of antimicrobials as beta-lactams, fluoroquinolones, and aminoglycosides.

Previous studies carried out in Colombia focused on *Acinetobacter spp.* have shown high resistance to beta-lactams, aminoglycosides and quinolones, and an increasingly and concerning resistance to carbapenems.[9,10]

The purpose of this study was to establish by using molecular approaches, the species from representative ACB complex strains isolated from patients hospitalized in four Colombian hospitals, their antibiotic resistance patterns and the presence of genes associated with resistance to four different groups of antibiotics.

Methods

From an initial collection of 150 isolates obtained during 2004, 2005, 2007 and 2009 from hospitalized patients in 4 Colombian hospitals, we selected 60 isolates previously identified by automated systems (Vitek Biomérieux, Marcy-l'Etoile, France) as *ACB* complex isolates and with diverse antibiotic-resistance patterns. Isolates were tested for susceptibility to cefotaxime, ceftazidime, cefepime, imipenem, meropenem, aztreonam, ampicillin–sulbactam, piperacillin–tazobactam, amoxicillin–clavulanic acid, ciprofloxacin, amikacin, gentamicin and nalidixic acid by disk diffusion method, and using CLSI interpretive standards.[11]

All isolates were representatives of clones with less than 75% similarity as evaluated by repetitive extragenic palindromic PCR (REP-PCR). Some of them were representatives of clones with more than one isolates.

The 16S–23S rRNA internal transcribed spacer (ITS) region was amplified for each isolate and for the *A. baumannii* ATCC 19606 strain, according to the conditions established by Chang et al.[6] The partial sequence of the *rpoB* gene was also amplified for all isolates that were not identified as *A. baumannii* and for some isolates identified by ITS as *A. baumannii*, according to conditions previously established.[12]

Genes codifying for resistance to cephalosporins, carbapenems and aminoglycosides were detected by PCR (Table 1). The strategy established by Vila et al. was used for detecting possible mutations in the quinolone resistance-determining region (QRDR).[19,20] The association of IS*Aba*-1 sequences with bla_{OXA} and bla_{ADC} genes was also determined by PCR. Amplification was done with forward primer for insertion sequence and reverse primer for bla_{ADC} gene and bla_{OXA}, respectively. Sequencing service was contracted with an external third party (Macrogen, Korea). Sequencing was done by single primer extension with an ABI Prism 3730xI-PE Applied Biosystems platform and resulting sequences were compared with sthose available on the Genbank.

A. baumannii strain ATCC 19606 was used as a positive control in detection assays of the $bla_{OXA\text{-}51}$ and bla_{ADC} genes, the ITS region, the *rpoB* gene fragment as well as a positive control for the restriction of the QRDR region of both *gyrA* and *parC* genes. *Klebsiella pneumoniae* strain 363 (from our laboratory) was used as a positive control for bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genes.

PCR products from ITS region, *rpoB* gene, some resistance genes and *gyrA* and *parC* genes were sequenced in both chains in an external facility (Macrogen, Korea). Each sequence was compared with public databases using the BLAST tool (available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Fifty-one out of the sixty isolates were identified as *A. baumannii*, eight as *A. nosocomialis* and one isolate was identified as *A. pittii*. ITS sequences revealed 99% to 100% similarity with those deposited in Genbank.

Isolates displayed different resistance patterns (Table 2). Antibiotics were grouped into six categories as follows: beta-lactams (cefotaxime, ceftazidime, cefepime, and aztreonam); carbapenems (imipenem, meropenem); beta-lactam/inhibitor combination (ampicillin–sulbactam, piperacillin–tazobactam, amoxicillin–clavulanic acid); quinolones (ciprofloxacin); aminoglycosides (amikacin, gentamicin) and nalidixic acid. According with definitions by Magiorakos et al.,[21] 46 isolates (all *A. nosocomialis*, the *A. pittii* and 37 *A. baumannii*) were classified as multidrug-resistant (MDR), 3 *A. baumannii* as extensively drug-resistant (XDR) and 10 *A. baumannii* as pandrug-resistant (PDR). Only one *A. baumannii* isolate was sensitive to all antibiotics tested except for nalidixic acid.

Table 1
Primers used for detection of resistance-related genes.

Genes	Primers 5′–3′	Annealing temperature (°C)	References
bla_{ADC}	ADC-7F: ATGCGATTTAAAAAAATTTCTTGT ADC-7R: TTATTTCTTTATTGCATTCAG	55	12
bla_{SHV}	SHV F: ATGCGTTATATTCGCCTGTG SHV R: TGCTTTGTTATTCGGGCCAA	58	13
bla_{TEM}	TEM F: AAACGCTGGTGAAAGTA TEM R: AGCGATCTGTCTAT	48	13
bla_{CTX-M}	CTX-MA: CGCTTTGCGATGTGCAG CTX-MB: ACCGCGATATCGTTGGT	55	14
bla_{VIM}	VIM1-F GTTAAAAGTTATTAGTAGTTTATTG VIM1-R CTACTCGGCGACTGAGC VIM2-F ATGTTCAAACTTTTGAGTAAG VIM2-R CTACTCAACGACTGAGCG	57	15
bla_{IMP}	IMP1-F ATGAGCAAGTTATCTGTATTC IMP1-R TTAGTTGCTTGGTTTTGATGG IMP2-F ATGAAGAAATTATTTGTTTTATG IMP2-R TTAGTTACTTGGCTGTGATG	57	15
bla_{OXA-23}	F: GATCGGATTGGAGAACCAGA R: ATTTCTGACCGCATTTCCAT	53	16
bla_{OXA-24}	F: GGTTAGTTGGCCCCCTTAAA R: AGTTGAGCGAAAAGGGGATT	53	16
bla_{OXA-51}	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	53	16
bla_{OXA-58}	F: AAGTATTGGGGCTTGTGCTG R: CCCCTCTGCGCTCTACATAC	53	16
*aac*A4	aacA4F:ATGACTGAGCATGACCTTGCG aacA4R:TTAGGCATCACTGCGTGTTCG	65	12
*aac*C1	aacC1-5′:ATGGGCATCATTCGCACATGTAGG aacC1-3′:TTAGGTGGCGGTACTTGGGTC	64	12
*aph*A6	aphA6 F: ATGGAATTGCCCAATATTATTC aphA6 R: TCAATTCAATTCATCAAGTTTTA	55	12
*aac*C2	aacC2F:ATGCATACGCGGAAGGCAATAAC aacC2 R: CTAACCGGAAGGCTCGCAAG	65	12
*arm*A	armA-F: ATTCTGCCTATCCTAATTGG armA-R:ACCTATACTTTATCGTCGTC	52	17
*gyr*A	gyrA-1: AAATCTGCCCGTGTCGTTGGT gyrA-2: GCCATACCTACGGCGATACC	55	18
*par*C	parC-1: AAACCTGTTCAGCGCCGCATT parC-2: AAAGTTGTCTTGCCATTCACT	55	19
IS*Aba*-1	ISab1 F: ATGCAGCGCTTCTTTGCAGG	55	20

All *A. nosocomialis* were resistant to ceftazidime and aztreonam and susceptible to ampicillin/sulbactam and ciprofloxacin. *A. pittii* was resistant to five antibiotics and was sensitive to ceftazidime, cefepime, ampicillin/sulbactam, imipenem, meropenem and ciprofloxacin.

Regarding the betalactams resistance, genes detected were bla_{ADC}, bla_{TEM}, and bla_{OXA}. The bla_{ADC} gene was identified in all *A. baumannii* isolates (Table 3). Thirty-eight out of the 44 ceftazidime-resistant isolates had the IS*Aba-1* sequence upstream of this gene. The remaining (6/44) had the bla_{ADC} gene but no association with IS*Aba-1*. Analysis of amplified bla_{ADC} sequences exhibited 100% of similarity with the bla_{ADC-5} gene. Only one of the *A. nosocomialis* strains had the bla_{ADC} gene, but this was not associated with the IS*Aba-1* sequence. This gene was not detected in *A. pittii*. bla_{TEM} genes were detected in 40 *A. baumannii* and in 2 *A. nosocomialis* isolates. The narrow spectrum TEM-1 enzyme-encoding gene was identified by sequence in some of these isolates. bla_{OXA} genes from families 23, 51 and 58 were detected in the isolates studied; bla_{OXA-23} was detected in *A. baumannii* and *A.nosocomialis* and was found to be associated with the IS*Aba-1* sequence in all carbapenem-resistant isolates. bla_{OXA-23} was also detected in a carbapenem-sensitive *A. nosocomialis* strain, but the IS*Aba-1* sequence was not found upstream of the gene.

Table 2
Antibiotic – resistance profile in *Acinetobacter* sp.

	Isolates per species			
	A. baumannii $n = 51$ (%)	*A. nosocomialis* $n = 8$ (%)	*A. pittii* $n = 1$ (%)	Total resistant isolates $n = 60$ (%)
Antibiotic				
Cefotaxime	50 (98)	8 (100)	1 (100)	59 (98)
Ceftazidime	44 (86)	8 (100)	0	52 (86)
Cefepime	43 (84)	4 (50)	0	47 (78)
Piperacillin/tazobactam	44 (86)	4 (50)	1 (100)	49 (81)
Ampicillin/sulbactam	40 (78)	0	0	40 (66)
Aztreonam	49 (96)	6 (75)	1 (100)	56 (93)
Imipenem	39 (76)	3 (37)	0	42 (70)
Meropenem	39 (76)	3 (37)	0	42 (70)
Ciprofloxacin	44 (86)	0	0	44 (73)
Amikacin	35 (68)	4 (50)	1 (100)	40 (66)
Gentamicin	42 (82)	5 (62)	1 (100)	48 (80)

Table 3
Resistance genes and QRDR mutations in *Acinetobacter* sp.

	A. baumannii (*n* = 51)	*A. nosocomialis* (*n* = 8)	*A. pittii* (*n* = 1)	Genes (*n* = 60) (%)
Antibiotic				
Cephalosporins				
*bla*TEM	40	2		42 (70)
*bla*ADC	51	1		52 (87)
IS*Aba*1-ADC	38			38 (63)
*bla*OXA-23	39	4		43 (72)
*bla*OXA-51	51			51 (85)
*bla*OXA-58			1	1 (2)
IS*Aba*1-OXA-23	39	3		42 (70)
Aminoglycosides				
aacC1	11	1		12 (20)
aacC2	44	5	1	50 (83)
aphA6		4	1	5 (8)
Quinolones				
gyrA Ser 83	44			44 (73)
par C Ser 80	44			44 (73)

The $bla_{\text{OXA-51}}$ gene was only detected in *A. baumannii* and $bla_{\text{OXA-58}}$ only in *A. pittii*. Those were not associated with IS*Aba-1*.

Genes encoding aminoglycoside-modifying enzymes were detected: *aac*C2 (83.3%), *aac*C1 (20%) and *aph*A6 (8.3%). The *aac*C2 gene was found in members of the three species, most of which were resistant to gentamicin. The *aph*A6 gene was found exclusively in those amikacin-resistant species other than *baumannii.*

The 44 ciprofloxacin-resistant *A. baumannii* isolates presented mutations in codons 83 and 80 of *gyr*A and *par*C genes, respectively. The sequences of some of the amplified products which were not digested with *Hin*f1 had Ser 83 to Leu changes in *gyr*A and Ser 83 to Tyr changes in *par*C.

Discussion

Some authors consider that identification to the species level is necessary for monitoring resistance in each species and for establishing possible differences in clinical manifestations and outcomes for patients infected by *Acinetobacter* sp. belonging to the ACB complex.[22]

A. baumannii was the most frequently genomic species detected in the four hospitals studied, followed by *A. nosocomialis.* Only one isolate was identified as *A. pittii*. We found differences in resistance patterns according with the species in our study. *A. baumannii* isolates exhibited the three resistance categories (MDR, XDR and PDR), while some *A. nosocomialis* were susceptible to cefepime, beta-lactams/inhibitor combination, aminoglycosides and ciprofloxacin. Ciprofloxacin and ampicillin/sulbactam susceptibility observed in *A. nosocomialis* may give an option for the treatment of infections caused by this microorganism. This susceptibility in species other than *baumannii* coincided with that observed in other investigations.[21,22] Presence of *aph*A6 gene exclusively in amikacin-resistant isolates of *A. nosocomialis* and *A. pittii* contrasted with what has been observed in other studies, where this gene has been found in a high percentage of *A. baumannii*.[23] Resistance to third-generation cephalosporins was related to the IS*Aba*-1-bla_{ADC} association in most of the isolates. This association has been especially documented in *A. baumannii* where such sequence provides a strong promoter for the overexpression of this gene.[24,25]

All the imipenem and meropenem resistant isolates had the IS*Aba*-1-$bla_{\text{OXA 23}}$ association. This constitutes the most disseminated mechanism of resistance to carbapenems in *Acinetobacter* sp. and some studies have shown that this association is enough to achieve resistance to this group of antibiotics. One *A. nosocomialis* that was sensitive to imipenem had the gene but it was not associated with the insertion sequence, thereby reaffirming the role of this IS in the overexpression of genes downstream of it.[25]

The $bla_{\text{OXA-51}}$ gene was only identified in *A. baumannii* consistently with established by some authors who consider such gene to be species-specific in *A. baumannii*.[22,26]

The $bla_{\text{OXA-58}}$ gene, was detected for the first time in Colombia only in the *A. pittii* identified in this study, although it has been previously detected in this species.[27,28] The detection of OXA carbapenemases has been rare in species other than *A. baumannii* in previous studies.[22] In this study the bla_{OXA} genes were detected in the other two species.

All the ciprofloxacin-resistant *A. baumannii* strains presented mutations in QRDR codons 83 and 80 in *gyr*A and *par*C genes and this is the main mechanism associated with resistance to quinolones as previously reported in other studies.[19,20]

High resistance to aminoglycosides was observed in the three species and the *aac*C2 gene was associated with resistance to gentamicin. The *aac*C1 gene was detected only in resistant isolates and concomitantly with *aac*C2 genes. The *aph*A6 gene, which confers resistance to amikacin and gentamicin, was only found in *A. nosocomialis* and *A. pittii* that were resistant to both gentamicin and amikacin. Resistance to amikacin in *A. baumannii* however, could not be explained with this gene evaluation. Other genes encoding aminoglycoside-modifying enzymes, as well as other non-enzymatic mechanisms, could be responsible for resistance to amikacin in *A. baumannii*. The absence of *aph*A6 gene in our *A. baumannii* strains contrasted with those from European clones I and II where this gene is found together with other aminoglycoside-resistant ones.[23]

This is the first report in Colombia that use molecular methods for differentiating species from the ACB complex and establish the presence of *A.nosocomialis* and *A. pittii* in hospitals in Colombia. The differences found in species susceptibility and molecular basis of that resistance, lead us to recommend that molecular techniques should be implemented in studies of surveillance of antibiotic resistance in members of the ACB complex.

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Conflicts of interest

Authors state that there does not exist any personal or financial relationship that may have caused a conflict of interest regarding this manuscript.

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