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IMIPENEM-RESISTANT *ACINETOBACTER BAUMANNII* CARRYING THE *ISABA1-BLA*$_{\text{OXA-23, 51}}$ AND *ISABA1-BLA*$_{\text{ADC-7}}$ GENES IN MONTERIA, COLOMBIA

Pedro Martínez and Salim Mattar*

Universidad de Córdoba. Instituto de Investigaciones Biológicas del Trópico. Montería, Colombia.

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ABSTRACT

The purpose of this study was to identify the genes coding for resistance to ceftazidime and imipenem and describe the molecular epidemiology of *A. baumannii* strains isolated from a clinical center in Colombia. Twenty isolates of imipenem-resistant *A. baumannii* from an equal number of patients with nosocomial infections were obtained. Primers were used to amplify genes *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{ADC-7}}$. To detect insertion sequences *ISAba1*/*bla*$_{\text{OXA-23}}$, *ISAba1*/*bla*$_{\text{OXA-51}}$ and *ISAba1*/*bla*$_{\text{ADC-7}}$, mapping by PCR using combinations of reverse primers *ISAba1* and reverse primers of *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{ADC-7}}$ were used. The amplification products were purified and cloned into PCR 2.1-TOPO vector and transformed into chemically competent *Escherichia coli* TOP10. These amplicons were then sequenced. PFGE was performed on DNA of *A. baumannii* isolates digested with *ApaI*. Results. The DNA profiles obtained included 9 clusters with, four 2-7 isolates per profile, and 5 single-isolate profiles. Of the 20 isolates resistant to imipenem, 15 carried *bla*$_{\text{OXA-23}}$ gene, 4 contained *ISAba1* upstream of *bla*$_{\text{OXA-51}}$ gene, and 6 contained *ISAba1* upstream of *bla*$_{\text{OXA-23}}$ gene. Eighteen of these isolates carried the *bla*$_{\text{ADC-7}}$ gene, with 9 of the isolates having *ISAba1* located upstream of this gene. This is the first report of the *ISAba1*/ADC-7 associated with OXAs genes in *A. baumannii* isolates from Colombia.

Key words: Nosocomial pathogens, Antimicrobial resistance, PFGE.

INTRODUCTION

Acinetobacter baumannii can cause a variety of infections including pneumonia, bacteremia, meningitis, urinary tract infections, peritonitis and infections of skin and soft tissue (2). Mortality is high in association with bacteremia (52%) and pneumonia (23-73% (6, 8). Multidrug resistant *A. baumannii* have become an important nosocomial pathogen that particularly affects critically ill patients. The multiresistance is common in this species which complicates its elimination and therapy in severe infections, with extremely limited therapeutic alternatives currently available (10, 30).

A. baumannii resistant to carbapenems has been isolated in Europe, Asia, North and South America (15). Although carbapenemase production, modification of penicillin-binding proteins (PBPs), loss of porins, and/or altered efflux pump activity are reported as mechanisms contributing to resistance. It is the production of carbapenemases that are the main

***Corresponding Author. Mailing address:** Universidad de Córdoba. Instituto de Investigaciones Biológicas del Trópico. Montería, Colombia.; Tel.: 57-47-569027.; E-mail: mattarsalim@hotmail.com

mechanism involved. These carbapenemases are mainly metalloenzymes (class B), found in several bacterial species of clinical relevance, including members of the family *Enterobacteriaceae*, *Pseudomonas* spp. and other non-fastidious Gram-negative nonfermenters. However, the vast majority of OXA carbapenemases (class D) have been discovered in *Acinetobacter baumannii* (18, 26).

In addition, the insertion sequence *ISAba1* has been found in many isolates of *A. baumannii* located upstream of the $bla_{\text{OXA-23,-51,-58}}$ carbapenemase genes and cephalosporinase bla_{ampC} genes. These *ISAba1* elements facilitate increased expression of these genes (4, 12). *ISAba1* belongs to the family of IS4 insertion sequences and possesses two imperfect inverted repeats of 16 bp. Its transposase is encoded by two open reading frames encoding 189 and 178 amino acids, leading to a functional protein when a frameshift occurs during the translation process (12). Over a 19-month period, it was observed imipenem-resistant *A. baumannii* isolates in intensive care units of a tertiary care clinic in Monteria-Colombia. The aim of this study was to describe the presence of the ISAba1/ADC-7, which is a novel class C enzyme, associated with *oxa* genes in *A. baumannii* isolates from Colombia.

MATERIALS AND METHODS

Bacterial strains

A. baumannii used in this study were collected from a private, tertiary care clinic in Monteria between August 2005 and February 2007. Study strains included 20 isolates resistant to imipenem obtained from an equal number of patients. The identification of *A. baumannii* was performed using Microscan Neg Combo Panel Type 44 (Dade Behring, Ca, USA). The isolates were obtained from adult (n = 14) and neonatal intensive care units (n = 6).

Determination of breakpoints

Antimicrobial compounds including imipenem, meropenem, ceftazidime, cefepime, aztreonam, amikacin, gentamicin, ciprofloxacin, moxifloxacin, ampicillin/sulbactam and piperacillin/tazobactam were used to establish the breakpoints. Breakpoints were determined by Microscan Neg Combo Panel Type 44 and interpreted according to CLSI standards (3). *Escherichia coli* ATCC® 25922 and *Pseudomonas aeruginosa* ATCC® 27853 were used as controls.

PCR, cloning and DNA sequencing

Screening of carbapenems and cephalosporins-resistant *A. baumannii* isolates was performed by PCR assay for genes $bla_{\text{OXA-51}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, bla_{IMP}, bla_{VIM}, and $bla_{\text{ADC-7}}$ using the primers described elsewhere (13, 14, 17, 19, 33, 34). The presence of the IS*Aba1* insertion upstream of OXA-23, OXA-51, and ADC-7, were determined by mapping using PCR with cloning and sequencing of products. Combinations of primers reverse *ISAba1* and reverse of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-51}}$ and $bla_{\text{ADC-7}}$ to detect *ISAba1*/$bla_{\text{OXA-23}}$, *ISAba1*/$bla_{\text{OXA-51}}$ and *ISAba1*/$bla_{\text{ADC-7}}$ were used for this purpose. Amplicons were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to manufacturer's instructions and cloned in PCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). These clones were subsequently transformed into chemically competent *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) by heat shock and selected on MacConkey agar containing ampicillin 50 mg/L. Plasmid DNA from transconjugantes was purified using Pure link™ quick plasmid miniprep Kit (Invitrogen, Carlsbad, CA, USA), and both strands were sequenced using an automatic DNA sequencer (MegaBACE 750, Amersham, Biosciences, Piscataway, NJ, USA). BLASTX search engine was used for the initial analysis of sequences (1) and alignments were created using Clustal W version 2.0.8 (29).

PFGE

Purified DNA from all isolates was prepared in agarose blocks and digested with 30U *ApaI* (Promega, Madison, WI) as described previously (23). Agarose gel electrophoresis was performed in 1% gels (Seakem Gold, Cambrex, USA) in 0.5 X

TBE buffer in an orthogonal-field alternating gel electrophoresis Gene Navigator (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 20 hours at 12ºC. The run conditions were 200 V with a pulse angle of 120° and pulse times of three phases as follows: 20s for 8h, 10s for 8h and 5s for 4h. A λ ladder (New England Biolabs) was used to provide molecular size markers, and the gels were stained with ethidium bromide (0.5 mg/L). Restricted DNA bands were visualized using a GE Healthcare imager (ImageQuant 100, Uppsala, Sweden). PFGE profiles were interpreted according to the criteria of Tenover *et al.* (28).

RESULTS

Phenotypic resistance

Breakpoint determinations and molecular detection of carbapenemases and cephalosporinase genes are shown in Table 1. All 20 *A. baumannii* isolates were resistant to imipenem and 18 to meropenem. Twelve and 17 isolates were resistant to fluoroquinolones and aminoglycosides, respectively, and all were resistant to the piperacillin/tazobactam and cephalosporins tested with the exception of ampicillin/ sulbactam, with 13 resistant isolates.

Table 1. Carbapenem resistance in relation to presence and location of *ISAba1,* and the PFGE profile.

MIC (µg/ml)									
CAZ	FEP	IPM	MER	*ISAba1*	Genes OXA, ADC-7	*IS*R/OXA51R	*IS*R/OXA23R	*IS*R/ADC-7R	PFGE profile
> 16	> 16	> 8	> 8	Negative	23,51, ADC-7	Negative	Negative	Negative	I
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Positive	Positive	I
> 16	> 16	8	< 4	Positive	51, ADC-7*	Negative	Negative	Negative	I
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Positive	Positive	I
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Negative	Negative	I
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Positive	Positive	I
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Negative	Negative	I
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Negative	Positive	II
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Negative	Positive	II
> 16	> 16	> 8	> 8	Positive	51, ADC-7	Positive	Negative	Positive	III
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Negative	Positive	III
> 16	> 16	> 8	> 8	Positive	23,51 ADC-7	Negative	Negative	Negative	IV
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Positive	Negative	IV
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Positive	Negative	IV
> 16	> 16	> 8	> 8	Positive	51*	Positive	Negative	Negative	IV
> 16	> 16	> 8	> 8	Positive	23, 51, ADC-7	Positive	Negative	Positive	V
> 16	> 16	> 8	> 8	Positive	23, 51, ADC-7	Negative	Positive	Negative	VI
> 16	> 16	> 8	> 8	Positive	23,51, ADC-7	Negative	Negative	Negative	VII
> 16	> 16	> 8	> 8	Positive	51, ADC-7	Positive	Negative	Positive	VIII
> 16	> 16	8	< 4	Positive	51*	Negative	Negative	Negative	IX

*CAZ: Ceftazidime; FEP: Cefepime; IMP: Imipenem; MER: Meropenem; ISR: ISAba1*R; *Imipenem-resistant isolates were negative for $bla_{\text{OXA-23, -24, -58,}}$ and $bla_{\text{IMP, VIM.}}$

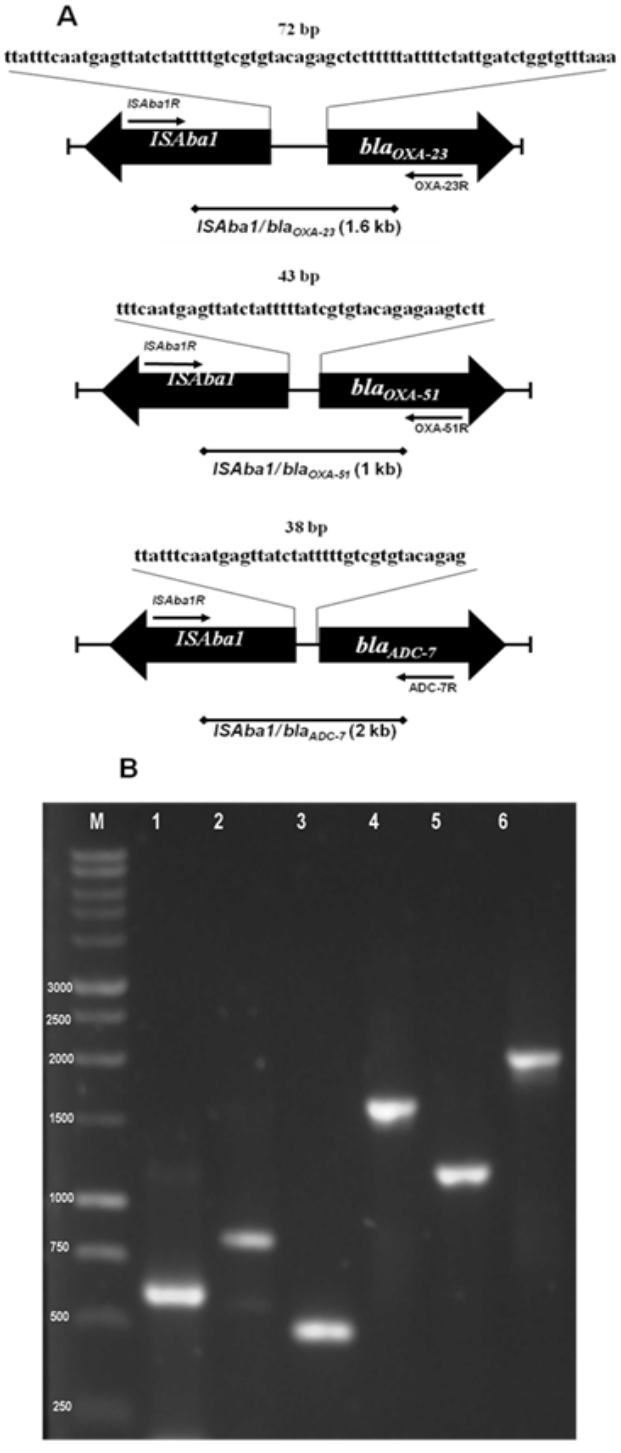
Detection of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-51}}$, and *ISAba1*/OXA-23-51

Amplification of purified DNA yielded a PCR product with specific primers for $bla_{\text{OXA-23,-51}}$ genes (Figure 1); the $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ genes were not detected by this method. The complete sequence of the fragment 507 bp was 100% identical to that described for the $bla_{\text{OXA-23}}$ gene (Accession number GU253894). The 641 bp fragment was identical to the $bla_{\text{OXA-51}}$ gene (Accession number EU255296).

Fifteen of the isolates were positive for the $bla_{\text{OXA-23}}$ gene, 4 others were producers of carbapenemase OXA-51 with the insertion of *ISAba1* in the promoter region of the $bla_{\text{OXA-51}}$ gene, and one of the 15 isolates carrying $bla_{\text{OXA-23}}$ also contained the *ISAba1*/OXA-51 gene. Two isolates were negative for carbapenemase genes (Table 1).

PCR mapping showed *ISAba1* to be located in the promoter region of the $bla_{\text{OXA-23}}$ gene in 6 isolates; these amplicons were 1.5-kb in size. The sequence obtained for the *ISAba1*-$bla_{\text{OXA-23}}$ amplicon was 97% identical to that described for the insertion sequence *ISAba1* transposase gene and the $bla_{\text{OXA-23}}$ gene (Accession number GU292795). Four other

isolates also had *ISAba1* located upstream of the $bla_{\text{OXA-51}}$ gene, and these amplicons were 1-kb in size (Figure 1). The sequence obtained the latter showed 96% identity to that described for the insertion sequence *ISAba1* transposase gene and $bla_{\text{OXA-51}}$ gene (Accession number HM545089)



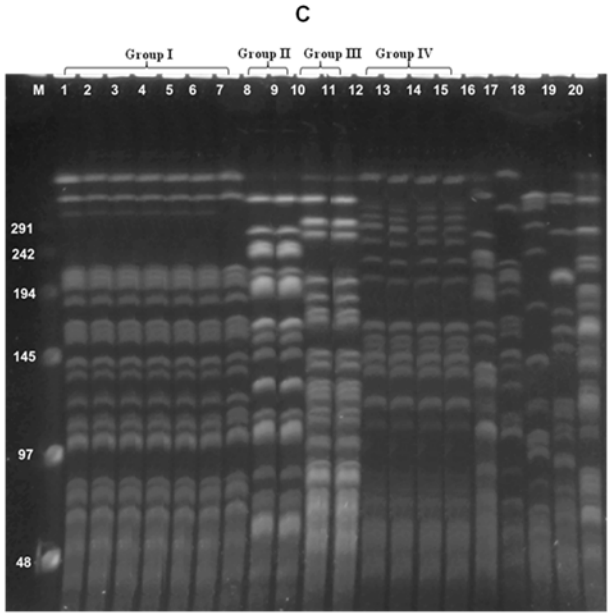


Figure 1. A. Location of the primers used in this work to amplify *ISAba1* located upstream of the genes $bla_{\text{OXA-23, 51}}$ and $bla_{\text{ADC-7}}$. B. PCR amplification of genes $bla_{\text{OXA-23, 51}}$ and $bla_{\text{ADC-7}}$. Line 1 OXA-51, line 2 ISAba1/OXA-51, Line 3 OXA-23, Line 4 ISAba1/OXA-23, Line 5 ADC-7, Line 6; ISAba1/ADC-7. C. PFGE profiles of 20 *A. baumannii* isolates digested with *ApaI* in lanes 1-20. Lane M, lambda DNA markers with molecular weights in kb shown.

Identification of $bla_{\text{ADC-7}}$ and *ISAba1*/ADC-7

All 20 isolates were resistant to third-and fourth-generation cephalosporins and piperacillin-tazobactam (Table 1). The PCR product for $bla_{\text{ADC-7}}$ produced an amplicon of 1.15-kb (Figure 1). Eighteen isolates contained the $bla_{\text{ADC-7}}$ gene. The nucleotide sequence of the 1.15-kb fragment was identical to the $bla_{\text{ADC-7}}$ gene (Accession number AY648950).

Mapping the position of *ISAba1* relative to $bla_{\text{ADC-7}}$.

Among the 18 isolates with $bla_{\text{ADC-7}}$ that were also resistant to carbapenems and cephalosporins, a band of 2-kb was obtained for 9 isolates using the reverse primer for *ISAba1* and the reverse primer for $bla_{\text{ADC-7}}$ (Figure 1 and Table 1). Sequencing showed 98% identity to that described for the insertion sequence *ISAba1* transposase gene and the β-lactamase ADC-7 (Accession number GU292796).

PFGE

Fifteen of the isolates clustered into four groups, while the remaining 5 isolates were unrelated to other strains and were categorized as having unique profiles. Group I contained seven isolates, groups II and III two isolates each, and group IV four isolates (Figure 1).

DISCUSSION

Carbapenems are the antibiotics of choice for the treatment of infections caused by *A. baumannii* when these bacteria are resistant to other β-lactam antibiotics. However, carbapenem resistance has increased, limiting the use of this class of agent for empiric antibiotic therapy (9). There is mounting evidence that *A. baumannii* has a natural intrinsic carbapenemase resistance mediated by $bla_{\text{OXA-51}}$ (27, 31). However, carbapenem resistance is only expressed when the insertion sequence IS*Aba1* is present upstream of the $bla_{\text{OXA-51}}$ gene (31).

Of the 20 carbapenem resistant *A. baumannii* isolates included in our study, 15 expressed the carbapenemase OXA-23 responsible for their carbapenem resistance. PFGE distinguished nine types clustered into four groups and involving 15 isolates. Although many nosocomial outbreaks are caused by a common clone (5, 24), the polymorphism observed mong isolates of this study suggest the existence of different lineages as a result of the mobilization of patients between clinics and hospital institutions.

Furthermore, studies of Villegas et al (32) have found widespread dissemination of OXA-23 among clinical isolates of *A. baumannii*, both clonal and non-clonal, in hospitals in several Colombian cities. Our data report similar findings and highlight the clinical and epidemiological problems associated with the existence of these resistant strains in the ICUs of hospitals due to the versatility of *A. baumannii* for nosocomial spread and contamination of the environment. Naturally this clinical picture complicates the choice of empirical antibiotic therapy in severely ill patients, even when using antibiotic alternatives such as polymyxins, minocycline and sulbactam (11).

The presence of the insertion sequence *ISAba1* upstream of carbapenemases genes can influence the expression of resistant genes (19). In our isolates containing $bla_{\text{OXA-51}}$, these were resistant to imipenem and meropenem if they possessed *ISAba1* upstream of $bla_{\text{OXA-51}}$ (Table 1).

In addition, 6 isolates that were positive for $bla_{\text{OXA-23}}$ gave a band of 1.5-kb using the ISAba1R and OXA-23R primers. Although these isolates were also positive for $bla_{\text{OXA-51,}}$ no amplicons were produced with primer combinations using ISAba1F/OXA-51R or ISAba1R/OXA-51R, suggesting that co-expression of these two carbapenemases genes was not occurring and that $bla_{\text{OXA-23}}$ alone was responsible for the carbapenem resistance phenotype of these strains.

Similarly, Poirel et al (16) found that expression of $bla_{\text{PER-1}}$ in *A. baumannii, P. aeruginosa* and *P. stuartii* was associated with the promoter sequence *ISPa12*. Futhermore, multiple copies of *ISAba1* are frequently found in *A. baumannii* isolates (21). These may be associated with several genes, as found in three isolates belonging to the same PFGE profile in this study, where *ISAba1* was associated with $bla_{\text{OXA-23}}$, and $bla_{\text{ADC-7}}$ (Table 1). Moreover, the PCR product amplified with the ISAba1R/ADC-7 reverse primers amplified a fragment of 2-kb in nine of the isolates, seven of these were also positive for $bla_{\text{OXA-23}}$, and 3 carried the promoter sequence *ISAba1* upstream of $bla_{\text{OXA-23}}$ gene (Table 1). Similar findings were reported by Héritier et al (12), who studied six isolates of *A. baumannii* resistant to ceftazidime and here these contained *ISAba1* upstream of the bla_{AmpC} gene; however, they did not find ISAba1/ADC in isolates positive for $bla_{\text{OXA-23}}$ and positive for *ISAba1*/OXA-23. As suggested by Segal et al (21), *ISAba1* could play an important role in the acquisition and expression of resistance genes.

However, Segal *et al.* (22) showed that transcription of the $bla_{\text{ADC-like}}$ gene is dependent on the promoter sequence within an *ISAba1* located upstream region of this gene. In the present study, the $bla_{\text{ADC-7}}$ gene was found upstream of *ISAba1* in 45%

of the isolates, which could be related to the regulation of this gene by activation or repression of resistance to third generation cephalosporins in isolates of *A. baumannii*. Similar results were reported by Corvec et al (4), who found *ISAba1* located upstream of the bla_{ADC} gene (no $bla_{\text{ADC-7}}$) in 52.4% of isolates of *A. baumannii*. However, Ruiz et al (19), also found *ISAba1* located upstream bla_{ADC} gene in 54% of the isolates of *A. baumannii*. Furthermore, *ISAba1* was located upstream from the bla_{ADC} gene in only one of the two *A. baumannii* strains belonging to the same clone, and was located upstream from the bla_{ADC} gene in only one of the two strains belonging to the same clone. In our study PFGE showed the presence of 9 profiles, with *ISAba1* upstream of the $bla_{\text{ADC-7}}$ gene in only 3 of 7 isolates belonging to profile 1. These results show the wide dissemination of *ISAba1* related to resistance genes in *A. baumannii* isolates in the hospital environment.

These findings highlight the emerging problem of carbapenem resistant *A. baumannii* in this Colombian hospital associated with OXA-23 and OXA-51 carbapenemases, and the links with *ISAba1* in some isolates. Two isolates that expressed resistance to imipenem but not meropenem and were negative for $bla_{\text{VIM, IMP, OXA23, OXA24, OXA58, and ADC}}$ genes (Table 1), and it is likely they have mutations in outer membrane porins or altered PBPs (7, 25).

In conclusion, this is the first report of the *ISAba1*/ADC-7 associated with OXAs genes in carbapenem-resistant *A. baumannii* isolates from Colombia. Furthermore, production of the OXA-23, ISAba1/OXA-23 and ISAba1/OXA-51 carbapenemases presents an emerging threat of carbapenem resistance among *A. baumannii* isolates which is particularly worrisome due to the difficult choice of empirical antibiotic therapy in seriously ill patients and the possible contribution to increased hospital stay and associated costs.

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