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Emergence and clonal dissemination of carbapenem-hydrolysing OXA-58-producing *Acinetobacter baumannii* isolates in Bolivia

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Acinetobacter baumannii is an emerging multidrug-resistant pathogen and very little information is available regarding its imipenem resistance in Latin American countries such as Bolivia. This study investigated the antimicrobial resistance profile of 46 clinical strains from different hospitals in Cochabamba, Bolivia, from March 2008 to July 2009, and the presence of carbapenemases as a mechanism of resistance to imipenem. Isolates were obtained from 46 patients (one isolate per patient; 30 males,16 females) with an age range of 1 day to 84 years, and were collected from different sample types, the majority from respiratory tract infections (17) and wounds (13). Resistance to imipenem was detected in 15 isolates collected from different hospitals of the city. These isolates grouped into the same genotype, named A, and were resistant to all antibiotics tested including imipenem, with susceptibility only to colistin. Experiments to detect carbapenemases revealed the presence of the OXA-58 carbapenemase. Further analysis revealed the location of the bla_{OXA-58} gene on a 40 kb plasmid. To our knowledge, this is the first report of carbapenem resistance in *A. baumannii* isolates from Bolivia that is conferred by the OXA-58 carbapenemase. The presence of this gene in a multidrug-resistant clone and its location within a plasmid is of great concern with regard to the spread of carbapenem-resistant *A. baumannii* in the hospital environment in Bolivia.

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INTRODUCTION

Acinetobacter baumannii is a nosocomial pathogen responsible for severe infections often diagnosed in patients attending hospitals worldwide. Carbapemens are considered the drugs of choice for treating these infections, but they are not always efficient due to the occurrence of multidrug-resistant strains of this bacterium (Canduela *et al.*, 2006; Coelho *et al.*, 2006; Higgins *et al.*, 2010a; Mendes *et al.*, 2009; Poirel *et al.*, 2010). Mechanisms enabling the development of carbapenem resistance include an induced decrease in membrane permeability, efflux pump overexpression and the production of carbapenemases. Among these mechanisms, the production of carbapenemases plays a major role in carbapenem resistance in most Gram-negative bacilli, including *A. baumannii* clinical isolates (Canduela *et al.*, 2006; Coelho *et al.*, 2006; Poirel *et al.*, 2010; Queenan & Bush, 2007; Walsh, 2010). The most common type of enzyme occurring in *A. baumannii* is represented by the carbapenem-hydrolysing class D β-lactamases, which are divided into five phylogenetic groups: OXA-23-like, OXA-24-like, OXA-58-like, OXA-51-like, which is intrinsic to *A. baumannii*, and the recently described OXA-143-like enzymes (Higgins *et al.*, 2009; Queenan & Bush, 2007).

Although *A. baumannii* is considered an emerging pathogen and much research has been carried out on it, there have been only a few studies concerning the mechanisms conferring carbapenem resistance of this bacterium in Latin American countries (Celenza *et al.*, 2006; Coelho *et al.*, 2006; Sader *et al.*, 2004; Sgambatti *et al.*, 2010). In addition, there are no previous publications regarding the resistance of *A. baumannii* to imipenem or the occurrence of carbapenemases in *A. baumannii* isolates in Bolivia.

In this study, we analysed the antibiotic resistance, in particular to imipenem, of clinical isolates of *A. baumannii* obtained from several hospitals in Cochabamba, Bolivia, and the presence of carbapenemases as a mechanism of resistance to imipenem in these strains.

METHODS

Bacterial isolates. Forty-three *A. baumannii* and three *Acinetobacter* genomic species 13TU, collected from March 2008 to July 2009 in the

Microbiology Service of the Hospital Gastroenterológico Boliviano-Japonés in Cochabamba, Bolivia, were analysed in this study. This hospital collects isolates from different hospitals of the city of Cochabamba: Hospital Viedma, Clínica Olivos and Hospital Materno-Infantil. They were identified using the API 32 system (bioMérieux) and the Phoenix System (Beckton Dickinson). Identification to species level was further confirmed by *gyrB* multiplex PCR (Higgins *et al.*, 2010c).

Isolates were obtained from 46 patients (one isolate per patient), comprising 30 males and 16 females of different ages ranging from 1 day to 84 years, and were collected following conventional bacteriological culture techniques from different samples: 17 specimens from respiratory tract infections, 13 from wounds, four from blood cultures, four from catheter samples, three from urine samples and five from other samples.

Susceptibility testing. MICs were determined using the Phoenix System (Beckton Dickinson) according to current Clinical and Laboratory Standards Institute guidelines (CLSI, 2007). The antibiotics tested were: amikacin, gentamicin, tobramycin, trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, ampicillin, aztreonam, cefazolin, cefepime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, colistin, imipenem, meropenem, piperacillin/tazobactam, rifampicin, fosfomycin, nitrofurantoin and ciprofloxacin.

Molecular typing by PFGE. *Apa*I-digested genomic DNA was prepared according to the manufacturer's instructions (Bio-Rad) and restricted fragments were separated on a CHEF-DR II system (Bio-Rad) for 30 h at 14 °C with 5–35 s of linear ramping at 6 V cm^{-1} (Turton *et al.*, 2004). DNA fingerprints were analysed using Quantity One 1-D Analysis Software (Bio-Rad) following criteria described previously (Tenover *et al.*, 1995).

β-Lactamase detection and sequencing experiments. Phenotypic detection of carbapenemases and metallo-β-lactamases was analysed by the Hodge test, and the Hodge test with zinc sulfate/double disc synergy test, respectively (Lee *et al.*, 2003). Extended-spectrum β-lactamases were determined by a double disc synergy test analysing the presence of synergy between discs of cephalosporins and inhibitors (Celenza *et al.*, 2006).

Genotypic detection of carbapenemase genes was carried out by multiplex PCR amplifying bla_{OXA-23}-like, bla_{OXA-24}-like, bla_{OXA-51}-like, bla_{OXA-58}-like and $bla_{OXA-143}$-like genes, and by PCR using primers specific for the bla_{IMP} and bla_{VIM} genes, as described previously (Canduela *et al.*, 2006; Higgins *et al.* 2010b; Woodford *et al.*, 2006). Fragments corresponding to the entire coding region of bla_{OXA-58} were sent for sequencing.

Presence of genetic class 1 integrons, insertion sequences and plasmids. PCR experiments were carried out to detect class 1 integrons (with primers corresponding to 3′ and 5′ conserved sequences) and insertion sequences IS*Aba-1*, *-2* and *-3* with specific primers, as described previously (Gallego & Towner, 2001; Poirel *et al.*, 2005). The relationship between bla_{OXA-58} and the detected insertion sequences was determined using custom primers anchored in the oxacillinase-encoding genes and in the IS elements.

Extraction of plasmid DNA was carried out using a commercial kit following the manufacturer's instructions (Plasmid Midi kit; Qiagen). The size of the plasmids was determined by comparison with control strains of *Escherichia coli* NCTC 50193 and NCTC 59192, which contain plasmids ranging in size from 2 to 163.3 kb, as described previously (Sevillano *et al.*, 2009).

Location and characterization of the bla_{OXA-58} gene. Plasmid extractions separated on agarose gels were transferred to a nylon membrane and subsequently UV cross-linked and hybridized with a specific digoxygenin–dUTP-labelled bla_{OXA-58} probe (Sambrook & Russell, 2001).

RESULTS AND DISCUSSION

Determination of MICs showed that there was a high level of resistance to all antibiotics tested, with the exception of colistin. All isolates were resistant to aztreonam, cefazolin, cefoxitin, cefuroxime, fosfomycin and nitrofurantoin, and levels of resistance >75 % were detected to most of the other antibiotics tested. In contrast, all isolates remained susceptible to colistin (Table 1).

In our study, imipenem resistance was observed in 35 % of isolates. This value was different from that obtained in a previous study carried out in hospitals in Santa Cruz (Bolivia), where carbapenem resistance was not found (Celenza *et al.*, 2006). It was difficult to compare the results in this study with those in other regions due to the lack of published reports. However, the observed percentage of carbapenem-resistant isolates was similar to that found in 2004 in Argentina (39.3 %), Venezuela (36.4 %), Brazil (31.1 %) and Colombia (30.8 %) (Rossi *et al.*, 2008; Sader *et al.*, 2004; Sgambatti *et al.*, 2010; Tognim *et al.*, 2004). In contrast, our results were different from those found for carbapenem resistance in isolates in Chile (6.5 %), Mexico (1.1 %) or Guatemala, Honduras, Panama, Puerto Rico and Jamaica where no carbapenem-resistant isolates were found (Tognim *et al.*, 2004). These percentages may have increased more recently as reported from hospitals in Puerto Rico, where imipenem-resistant isolates have emerged recently (Robledo *et al.*, 2010). Reports from Asia-Pacific nations and Europe have revealed on average a lower percentage of carbapenem-resistant isolates (Mendes *et al.*, 2009; Souli *et al.*, 2008).

The clonal relatedness of the isolates enabled us to group them into four genotypes named A (15 isolates), B (eight isolates), C (nine isolates) and D (four isolates), in addition to unrelated single isolates. Comparisons of PFGE patterns with those from control strains belonging to European clones I, II and III showed no genetic relatedness. Analysis of their antibiotic resistance showed that (i) isolates from clone A were resistant to all antibiotics tested with the exception of colistin to which they remained susceptible and (ii) the majority of isolates belonging to groups B, C and D were susceptible only to carbapenems and colistin.

Molecular typing results indicated inter-hospital dissemination of carbapenem-resistant clone A, as identical pulsotypes were identified in different institutions in Cochabamba. This fact is alarming, as there are only a few drugs that are clinically available to treat infections caused by this multidrug-resistant pathogen.

Class 1 integrons, ranging in size from 540 to 1100 bp, were present in all strains. The predominant band pattern was a combination of 540 and 780 bp structures present in 32 isolates, in turn belonging to different groups. The

Table 1. MICs (μg ml^{-1}) of antibiotics with *in vitro* activity against *A. baumannii* clinical isolates

All isolates were resistant to aztreonam, cefazolin, cefoxitin, cefuroxime, fosfomycin and nitrofurantoin. V, Viedma Hospital; G, Hospital Gastroenterológico Boliviano-Japonés; O, Clínica Olivos; M, Hospital Materno-Infantil.

Antibiotic*	Clone A (15 isolates: 11V, 2G, 2O)			Clone B (8 isolates: 6V, 2G)			Clone C (9 isolates: 6V, 3G)			Clone D (4 isolates: 3V, 1M)			Other clones (7 isolates: 3G, 2O, 2M)		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
AMK	>32	>32	>32–>32	⩽8	>32	⩽8–>32	32	>32	32–>32	>32	>32	>32–>32	⩽8	>32	⩽8–>32
GEN	>8	>8	>8–>8	>8	>8	>8–>8	>8	>8	4–>8	>8	>8	>8–>8	⩽2	>8	⩽2–>8
TOB	>8	>8	>8–>8	8	>8	⩽2–>8	>8	>8	⩽2–>8	8	>8	8–>8	⩽2	>8	⩽2–>8
SXT	>2/38	>2/38	1/19–>2/38	>2/38	>2/38	⩽0.5/0.9–2/38	>2/38	>2/38	>2/38–2/38	>2/38	>2/38	⩽0.5/0.9–>2/38	⩽0.5/0.9	>2/38	⩽0.5/0.9–>2/38
AMC	>16/8	>16/8	>16/8	>16/8	>16/8	16/8–>16/8	>16/8	>16/8	16/8–>16/8	>16/8	>16/8	>16/8–>16/8	16/8	>16/8	⩽4/2–>16/8
AMP	>16	>16	>16–>16	>16	>16	>16–>16	>16	>16	16–>16	>16	>16	>16–>16	16	>16	⩽4–>16
FEP	>16	>16	>16–>16	>16	>16	4–>16	>16	>16	4–>16	16	16	16–16	4	>16	⩽2–>16
CAZ	>16	>16	>16–>16	>16	>16	2–>16	>16	>16	4–>16	>16	>16	>16–>16	4	>16	2–>16
CRO	>32	>32	>32–>32	>32	>32	8–>32	>32	>32	16–>32	>32	>32	>32–>32	16	>32	4–>32
IPM	>8	>8	>8–>8	2	2	⩽1–2	⩽1	2	⩽1–2	2	2	2–2	⩽1	⩽1	⩽1–⩽1
MEM	>8	>8	8–>8	⩽1	8	⩽1–8	⩽1	2	⩽1–8	⩽1	4	⩽1–4	⩽1	⩽1	⩽1–⩽1
TZP	>64/4	>64/4	>64/4–>64/4	>64/4	>64/4	⩽4/4–>64/4	64/4	>64/4	16/4–>64/4	>64/4	>64/4	16/4–>64/4	⩽4/4	64/4	⩽4/4–>64/4
CIP	>2	>2	>2–>2	>2	>2	0.5–>2	>2	>2	>2–>2	>2	>2	>2–>2	1	>2	⩽0.125–>2
NOR	>8	>8	>8–>8	>8	>8	⩽2–>8	>8	>8	>8–>8	>8	>8	>8–>8	8	>8	⩽2–>8
CST	<1	<1	<1–2	⩽1	⩽1	⩽1–2	⩽1	⩽1	⩽1–⩽1	⩽1	⩽1	⩽1–⩽1	⩽1	⩽1	⩽1–⩽1
RIF	8	16	8–16	>128	>128	>128–>128	>128	>128	16–>128	16	>128	16–>128	16	16	8–>128

*AMK, Amikacin; GEN, gentamicin; TOB, tobramycin, SXT, trimethoprim/sulfamethoxazole; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; FEP, cefepime; CAZ, ceftazidime; CRO, ceftriaxone; IPM, imipenem; MEM, meropenem; TZP, piperacillin/tazobactam; CIP, ciprofloxacin; NOR, norfloxacin; CST, colistin; RIF, rifampicin.

presence of integrons occurring with high frequency in these clinical isolates could be related to the resistance to aminoglycosides, as the genes encoding aminoglycoside-inactivating enzymes are often found on these genetic elements worldwide (Gallego & Towner, 2001).

Plasmids were found in 35 isolates, ranging in size from 2 to 40 kb. The most frequently detected genetic element was a 40 kb plasmid present in 15 isolates in group A (Fig. 1). Other plasmids of 2.5 kb (13 isolates), 11 kb (seven isolates), 5.8 kb (five isolates), 9 kb (two isolates), 28 kb (two isolates) and 14 kb (one isolate) were also detected.

As imipenem is the last therapeutic option when treating nosocomial infections caused by antibiotic-resistant *A. baumannii* isolates, we investigated further whether the presence of β-lactamases potentially expressed by these isolates could confer their resistance to imipenem. No extended-spectrum β-lactamase-producing isolates were detected in this study, although $bla_{\text{CTX-M-type}}$ and $bla_{\text{PER-2}}$ genes were detected in a previous study carried out in Santa Cruz, another region of Bolivia (Celenza *et al.*, 2006). This suggests that resistance to cephalosporins could be due to other mechanisms, such as the expression of chromosomal ADC β-lactamase, reduced membrane permeability caused by a decrease in expression of porin genes and/or an increase in the efficiency of efflux pumps.

Carbapenemase activity was detected in 70 % of the isolates by the Hodge test, probably due to expression of the intrinsic OXA-51 carbapenemase or other acquired carbapenemases. Although they have been reported in *A. baumannii* from other Latin American countries (Sader *et al.*, 2005), further PCR experiments detected no metallo-β-lactamase genes in our isolates.

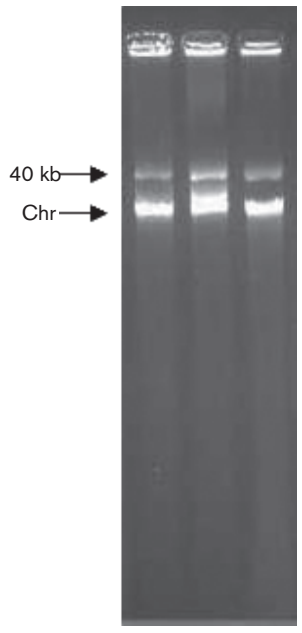

Fig. 1. Examples of the 40 kb plasmid DNA from different isolates belonging to clone A. The chromosomal DNA (Chr) is also indicated.

The multiplex PCR detected the presence of the $bla_{\text{OXA-51}}$-like gene in all *A. baumannii* strains except three isolates that were subsequently identified to be *Acinetobacter* 13TU by the *gyrB* multiplex PCR. In addition, an amplification band corresponding to the $bla_{\text{OXA-58}}$ gene was detected in all isolates of the multidrug-resistant clone A group. Sequencing of the 843 bp amplicon showed 100 % identity with the $bla_{\text{OXA-58}}$ gene sequence described previously (Poirel *et al.*, 2005) and the presence of an IS*Aba-3* element. Southern blot analysis with a $bla_{\text{OXA-58}}$-specific probe generated by PCR enabled us to localize this gene on the 40 kb plasmid. This plasmid was within the size range (10–150 kb) described by other authors (Bertini *et al.*, 2006; Héritier *et al.*, 2005; Marqué *et al.*, 2005; Poirel *et al.*, 2005; Wang *et al.*, 2007).

Although the $bla_{\text{OXA-58}}$ gene has also been detected in isolates from other Latin American countries, we believe that this is the first report of this gene in Bolivian clinical isolates. Our results support previous reports indicating the worldwide spread of the $bla_{\text{OXA-58}}$ gene and highlight its importance in conferring carbapenem resistance among *A. baumannii* isolates from Latin America (Coelho *et al.*, 2006; Higgins *et al.*, 2010a; Kulah *et al.*, 2010; Marqué *et al.*, 2005; McCracken *et al.*, 2009; Mendes *et al.*, 2009; Papa *et al.*, 2009).

In conclusion, this is the first report, to our knowledge, of carbapenem resistance in *A. baumannii* isolates from Bolivia associated with the presence of the $bla_{\text{OXA-58}}$ gene. Detection of the antibiotic-inactivating enzyme in a multidrug-resistant clone and its expression is of great concern with regard to the spread of carbapenem resistance among hospitals in Bolivia.

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