Multi-drug resistance profiles and the genetic features of Acinetobacter baumannii isolates from Bolivia

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Abstract
Introduction: Acinetobacter baumannii is opportunistic in debilitated hospitalised patients. Because information from some South American countries was previously lacking, this study examined the emergence of multi-resistant A. baumannii in three hospitals in Cochabamba, Bolivia, from 2008 to 2009. Methodology: Multiplex PCR was used to identify the main resistance genes in 15 multi-resistant A. baumannii isolates. RT-PCR was used to measure gene expression. The genetic environment of these genes was also analysed by PCR amplification and sequencing. Minimum inhibitory concentrations were determined for key antibiotics and some were determined in the presence of an efflux pump inhibitor, 1-(1-naphthylmethyl) piperazine. Results: Fourteen strains were found to be multi-resistant. Each strain was found to have the blaOXA-58 gene with the ISAba3-like element upstream, responsible for over-expression of the latter and subsequent carbapenem resistance. Similarly, ISAba1, upstream of the blaADC gene caused over-expression of the latter and cephalosporin resistance; mutations in the gyrA(Ser83 to Leu) and parC (Ser-80 to Phe) genes were commensurate with fluoroquinolone resistance. In addition, the adeA, adeB efflux genes were over-expressed. All 15 isolates were positive for at least two aminoglycoside resistance genes. Conclusion: This is one of the first reports analyzing the multi-drug resistance profile of A. baumannii strains isolated in Bolivia and shows that the over-expression of the blaOXA-58, blaADC and efflux genes together with aminoglycoside modifying enzymes and mutations in DNA topoisomerases are responsible for the multi-resistance of the bacteria and the subsequent difficulty in treating infections caused by them.

Key words: Acinetobacter baumannii; insertion sequences; beta-lactamases; carbapenems; gene; environment


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Introduction
Acinetobacter baumannii is a pathogenic bacterium responsible for a wide range of infections such as septicaemia, meningitis, pneumonia and urinary tract infections and is one of the most important Gram-negative pathogens causing infections in immuno-compromised patients [1]. Acinetobacter baumannii has been considered the paradigm of multi-resistant bacteria because of emerging multi-drug resistance to various antimicrobial agents [2], notably mutations in gyrA and parC genes that confer fluoroquinolone resistance and activation of the aminoglycoside inactivating enzymes [2,3,4]. Beta-lactam antibiotics (mainly carbapenems) are now the first drug of choice to treat these microorganisms; however, in the last decade, resistance to carbapenems has appeared in hospitals worldwide owing to the production of beta-lactamases, changes in permeability, increase in efflux, and modification of the affinity of penicillin-binding proteins (PBPs) in these bacteria [2,3].

Transposable elements also play a major role in gene expression. They are generally tightly regulated and exercise their role in a strategic manner [5]. Insertion sequences carrying promoters are often responsible for driving the expression of the downstream antibiotic resistance gene, often leading to the over-expression of the gene, and making the bacterium resistant to various antibiotics [6].

A. baumannii is considered an emerging pathogen but very few reports are available from developing countries in Latin America on the mechanisms responsible for carbapenem resistance. In this study we analysed the factors involved in antibiotic resistance in various clinical strains isolated from three major hospitals in Cochabamba, Bolivia. We also
investigated the genetic environments responsible for carbapenem and ceftazidine resistance as well as the mutations that confer resistance to fluoroquinolones.

Methodology
Fifteen isolates thought to have a multi-resistant profile were selected for this study. The isolates were obtained from three different hospitals in Cochabamba in 2008-2009 [Table]. The isolates were identified as *A. baumannii* by PCR amplification and sequencing of the bla*OXA*-51-like [7] and *rpoB* genes [8].

Antimicrobial susceptibility testing
All the isolates were tested for their susceptibility to imipenem (IPM), meropenem (MEM), ceftazidine (CAZ), ciprofloxacin (CIP), and gentamicin (CN). Minimum inhibitory concentrations (MICs) were determined by the agar double dilution method according to the British Society for Antimicrobial Chemotherapy (BSAC) methodology [9]. The MICs of ciprofloxacin and gentamicin were also determined in the presence of an efflux pump inhibitor 1-(1-naphthylmethyl), piperazine (NMP) (100mg/L). The results were interpreted according to BSAC guidelines [9]. The reference strains used for MIC testing were *Escherichia coli* NCTC 10418, *P. aeruginosa* ATCC 10662, and *S. aureus* NCTC 6571.

Screening for antimicrobial resistance genes of the *bla*~OXA~ family
All the isolates were screened for the presence of genes of the *bla*~OXA~23-like, *bla*~OXA~40-like, *bla*~OXA~51-like, *bla*~OXA~58-like and *bla*~OXA~143-like families by multiplex PCR described by Higgins and colleagues [10]. The primers described by Héritier et al. [11] were used for the amplification and sequencing of the *bla*~OXA~58-like gene. The primer SM2, described by Poirel and Nordmann [12], was used in combination with walk-58-R [13] for amplification and sequencing of the region upstream of the *bla*~OXA~58-like gene.

Screening for the disruption of the *carO* gene
PCR amplification for the insertions causing disruption of the *carO* gene (29kDa OMP) was also completed for all the isolates using primers as described by Mussi et al. [14].

Screening for the presence of aminoglycoside resistance genes
A multiplex PCR was performed for the screening of aminoglycoside resistance genes as described earlier by Noppe-Leclercq et al. [4]. PCR was also performed to check for the presence of the *armA* gene and other rRNA methylases such as *rmtA*, *rmtB* and *rmtC* using primers described by Yamane et al. [15]. The *rmtD* gene screening was completed using primers described by Doi et al. [16].

Screening for the presence of other antimicrobial determinants
PCR arrays were performed for the identification of *bla*~TEM~, *bla*~SHV~, *bla*~PER~ and *bla*~CTX-M-2~ enzymes using primers described by Celenza et al. [17]. Amplification of the *bla*~VEB~ and *bla*~GES~ genes was performed as described by Moubareck et al. [18].

Analysis of the genetic environment of the *bla*~ADC~ gene
PrimersFU (5'-GCGGCGTGGATCTTAAGTG-3') and RU (5'-AGCCCATACCTGCGACCATCAT-3') were used for amplification of the intergenic region upstream of the *bla*~ADC~ gene. Primers FD (5'-CAGCTTATGCTGCTGGAT-3') and RD (5'-GAGCTGCCATATTTGGGAAGA-3') were used to amplify the intergenic region downstream of the *bla*~ADC~ gene.

Analysis of the quinolone resistance-determining region (QRDR)
PCR assays as described by Valentine and colleagues [3] were used for the amplification of the QRDRs of the *gyrA* and *parC* genes. The gene fragments were sequenced and checked for specific amino acid changes.

Analysis of the *adeABC* and *adeRS* genes involved in efflux
PCR was performed to check for the presence of *adeABC* and *adeRS* genes. The intergenic regions of *adeRS* were amplified using the primers adeRF (5'-GCA TTA CGC ATA GGT GCA GA-3') and adeSR (5'-GAG GTC GGC GTG ACT AAT TT-3'). The primers adeRA (5'-TCA CGG GAG GAG TCT GCT TT-3') and adeAB (5'-AAT AGG CGC TCG AAC TGT TG-3') were used to check for any insertions between the *adeRA* genes. The primers adeAF (5'-CCG AAG TCG GAG GTA TCA TT-3') and adeAR (5'-TAT ACC TGA GGC TCG CCA CT-3') were used for the amplification of *adeA*, while the primers adeBF (5'-CCCTAATCAAGGACGTATGC-3') and adeBR (5'-TAG AGT GCA GCC AAG ACA AG-3') were used for the amplification of the *adeB* gene. The primers adeCF (5'-AGCCTGCAATTACATCTCAT-3') and adeCR (5'-TGGCACTTACATCAATAC-3') were used for the amplification of the *adeC* gene.
3’) were used to check for the presence of the adeC outer membrane gene.

Analysis of gene expression

Expression of the bla\textsubscript{ADC}, bla\textsubscript{OXA-58-like}, adeA, adeB and adeC genes was studied by RT-PCR. The primers for the bla\textsubscript{ADC} gene were those defined by Ruiz et al. [19] and those used for bla\textsubscript{OXA-58-like} gene expression were the multiplex primers described by Woodford et al. [20]. The adeAF and adeAR, adeBF and adeBR, and adeCF and adeCR primers were used to determine adeA, adeB, and adeC expression. Total RNA was extracted from isolates in the exponential growth phase using the RiboPure Bacteria kit (Ambion, Paisley, United Kingdom) and treated with the DNase I provided. cDNA was synthesised from 100ng of RNA using the Access quick RT-PCR system kit (Promega, Southampton, United Kingdom). The PCR products were quantified using the Bio-Rad Quantity One Software 4.6.1 (Bio-Rad, Hemel Hempstead, United Kingdom). 16S-rRNA gene primers as described by Lin et al. [21] were used for normalization. The results were confirmed three times and were based on the average of the mean increase or decrease of the individual strains.

Results

Isolates were confirmed as \textit{A. baumannii} by bla\textsubscript{OXA-51-like} (Table) and rpoB gene PCR. All strains except isolate 2 were resistant to one or more antibiotics tested. They had previously been confirmed to be clonally related according to their PFGE profiles [22].

The table shows the MIC values of IPM, MEM, CAZ, CIP, CIP+NMP, CN and CN+NMP in each individual isolate. It shows that all the strains except two were multi-resistant. The MIC of gentamicin did not change upon the addition of NMP; however, NMP did give a two- to four-fold decrease in isolates resistant to CIP, suggesting that efflux pumps partially contribute to resistance in these isolates.

The table identifies the specific bla\textsubscript{OXA-51-like} gene in each isolate; none of them had insertion sequences upstream of the bla\textsubscript{OXA-51-like} gene. All isolates except number 2 were positive for the bla\textsubscript{OXA-58-like} gene determined by multiplex PCR. Sequencing of the bla\textsubscript{OXA-58-like} gene revealed no nucleotide changes. The primers SM-2 and walk-58-R detected the insertion of an IS\textit{Abal}3-like structure 17bp upstream of the bla\textsubscript{OXA-58} gene. Putative promoters with -35 (TTRYTTC) and -10 (TTRYTCTT) motifs were detected in the IS\textit{Abal}3-like element. The promoters had previously been identified by Poirel and Nordman [11] and correlate with the high MICs of carbapenems resistance in these isolates (Table). The IS\textit{Abal}3-like element had a single codon change at position 25 (GAT to AAT) which led to an aspartate to asparagine amino acid change; however, this but did not affect its activity. The role of the insertion sequence is supported by the observation that the RT-PCR for the bla\textsubscript{OXA-58-like} gene expression also recorded high levels of expression in the isolates positive for the bla\textsubscript{OXA-58-like} gene (Table). The bla\textsubscript{OXA-58-like} gene was found to be present on the 40kb plasmid in all strains resistant to carbapenems except isolate 2 (which was carbapenem sensitive).

The \textit{carO} gene fragment was not disrupted by any insertion element (as indicated earlier by Mussi et al. [14]) and thus did not contribute to reduced carbapenem susceptibility.

Multiplex PCR revealed that all isolates except isolate 2 were positive for two aminoglycoside resistance enzymes, the \textit{aac(6′)-Ib} and \textit{aph(3′)-VI}. As reviewed by Shaw et al. [23] the \textit{aac(6′)-Ib} class of aminoglycoside inactivating enzymes has been shown to confer resistance to tobramycin, dibekacin, amikacin, 5-episomicin, netilmicin, 2′-N-ethylnetilmicin, and sisomicin, whereas the \textit{aph(3′)-VI} group of enzymes is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin, amikacin, isepamicin and gentamicin. Isolate 2, on the other hand, was positive for three enzymes: \textit{aph(3′)-Ia}, which confers resistance to various aminoglycosides including gentamicin; \textit{aac(3)-Ia}, responsible for resistance to gentamicin and fortimicin; and \textit{ant(2′)-Ia}, which hydrolyzes tobramycin, dibekacin, sisomicin, kanamycin and gentamicin. In each strain these combinations of enzymes contribute to the high MIC of gentamicin. The MICs of gentamicin did not decrease after the addition of NMP, indicating that resistance to gentamicin may not be efflux mediated. Aminoglycoside resistance is common in \textit{Acinetobacter} spp and often derives from inactivation of the antibiotic by the specific modifying enzymes described above [24]. The PCR assays for the rRNA methylase genes such as \textit{armA}, \textit{rmtA}, \textit{rmtB}, \textit{rmtC}, and \textit{rmtD} were all negative.

PCR performed for the identification of \textit{bla}\textsubscript{SHV}, \textit{bla}\textsubscript{PER-2}, \textit{bla}\textsubscript{CTX-M-2}, \textit{bla}\textsubscript{VEB} and \textit{bla}\textsubscript{GES} genes were negative. All isolates had the \textit{bla}\textsubscript{TEM-1} gene which was amplified and sequenced.

\textit{IS}\textit{Abal} was found to be present upstream of the bla\textsubscript{ADC} gene in all the strains except isolate 2; the latter had a low MIC of ceftazidime. There was no insertion
Table. *In vitro* activities of antibiotics, levels of gene expression and the mutations in QRDR’s in the *A. baumannii* clinical isolates

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<th>No</th>
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<th>CN+NMP</th>
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*GBJ = Gastroenterológico Boliviano-Japonés, V = Viedma, CO = Clinica Olivos*

*Relative gene expression was determined by RT PCR and quantified by Bio-Rad quantity I software.

+ = present

- = absent

IPM = imipenem, CN = gentamicin, CIP = ciprofloxacin, CAZ = ceftazidime, NMP = 1-(1-naphthylmethyl) piperazine

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detected downstream of the \textit{bla}_{ADC} gene in any of the isolates. Expression of the \textit{bla}_{ADC} gene in the strains was identified by the high MIC values of ceftazidime and confirmed by RT-PCR.

The intergenic regions of adeRS were amplified using the primers adeRF (5'-GCA TTA CGC ATA GGT GCA GA-3') and adeSR (5'-GAG GTC GCC GTG ACT AAT TT-3'); these amplified a 790bp product, indicating that there was no insertion. The primers adeRA (5'-TCA CCG GAG TCT GAG CTT TT-3') and adeAB (5'-AAT AGG CGC TCG AAC TGT TG-3') amplified a product of 929bp, indicating there was no insertion between these two genes. Ruzin \textit{et al.} [25] previously reported that IS\textit{Abal} may contribute to the expression of the efflux genes. The primers adeCF (5'-AGC CTG CCA TTA CAT CTC AT-3') and adeCR (5'-TGG CAC TTC ACT ATC AAT AC-3') detected the presence of the \textit{adeC} gene. Because the strains had a very high MIC of ciprofloxacin, which decreased after the addition of NMP (Table), we hypothesized that the efflux pump genes would be highly expressed. RT-PCR for the analysis of the \textit{adeA} (encoding for the membrane fusion protein) and the \textit{adeB} (encoding the RND-transporter) genes revealed that both the genes were highly expressed as they are co-transcribed (Table). The \textit{adeC} (encoding the outer membrane protein) was not as highly expressed as \textit{adeA} and \textit{adeB}; the reason may that its expression is not necessary to confer resistance [26] as alternative outer membrane proteins can be used by bacteria to export substrates for effluxing any poisons or antibiotics. We checked further for mutations in \textit{gyrA} and \textit{parC} as well as the antibiotic determinants responsible for ciprofloxacin resistance in these isolates. Sequencing of the \textit{gyrA} and \textit{parC} genes revealed Ser-83 to Leu and Ser-80 to Phe amino acid changes in isolates resistant to ciprofloxacin. Resistance to ciprofloxacin was mainly due to target site mutations in the \textit{gyrA} and \textit{parC} with a small contribution by the efflux pumps.

**Discussion**

This work analyzed the genetic environment and the multi-drug resistance profile of \textit{A. baumannii} isolated in Cochabamba, Bolivia, as there are few surveys that study the multi-drug resistant profile of clinical isolates in Bolivia. Multi-drug resistance is certainly of great concern. A previous study by Celenza \textit{et al.} [17] reported no carbapenem resistance in any of the isolates studied. The current investigation showed that carbapenem resistance is manifested largely by the presence of the OXA-58 carbapenemase. Surprisingly, the PCR results for the \textit{bla}_{PER-2} and \textit{bla}_{CTX-M-2} genes were negative. These results contradict those of a study conducted in Bolivia in 2006 by Celenza \textit{et al.} [17] in which the presence of these genes was reported. We therefore conclude that the presence of IS\textit{Abal} upstream of the \textit{bla}_{ADC} gene has substituted the \textit{bla}_{PER-2} and \textit{bla}_{CTX-M-2} genes to confer high levels of resistance to ceftazidime and provide a stable mechanism of cephalosporin resistance to the bacterium.

The presence of the \textit{bla}_{OXA-58} gene has been observed in other Latin American countries. These results support the worldwide spread of the \textit{bla}_{OXA-58} gene, one of the major causes of carbapenem resistance [27]. Ciprofloxacin resistance in the isolates was largely determined by target site mutations with partial contribution by the efflux pumps. Each strain had at least two aminoglycoside modifying enzymes which together contributed to high levels of resistance to gentamicin. The high proportion of strains with resistance genes to the major groups of antibiotics used to control \textit{A. baumannii} found in this study is of great concern as clonal spread has been observed among the three hospitals where these isolates were isolated. Mobilization of \textit{bla}_{OXA-58-like} carbapenemase genes has often been found to be associated with insertion sequences or transposons, and the potential of spread via plasmid motors is very high. The OXA-23-like and OXA-58-like enzymes have been found in Brazil, Venezuela, Colombia, Chile, Bolivia and Argentina; however, OXA-58-like enzymes are most frequently identified in South America [28]. The spread of plasmid-borne carbapenemases can occur inter- or intra-specifically in hospital environments, so it is important to develop control strategies to prevent infections caused by carbapenem-resistant \textit{A. baumannii}. This strategy should help in the prevention of nosocomial spread of infections, especially among immuno-compromised patients.

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