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Contribution of Outer Membrane Protein Alterations and ß-Lactamases in Carbapenem Resistance in *Acinetobacter* spp.

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SENTRY ANTIMICROBIAL SURVEILLANCE

AMENDED ABSTRACT

Objectives: As part of the SENTRY Program, *Acinetobacter* spp. isolates have been screened for resistance to carbapenems (imipenem [IMP] and meropenem [MER]) and ceftazidime (CTZ). Thirty-four isolates exhibiting this profile recovered in 2002 from Buenos Aires, Argentina were evaluated.

Methods: All isolates were tested against CTZ, IMP, MER with and without the serine-ß-lactamase inhibitor BRL 42715 or the AmpC inhibitor cloxacillin or the pump inhibitor reserpine by agar dilution. ß-Lactamase activity against IMP and MER was evaluated using standard spectroscopic techniques. The number of ß-lactamases was first evaluated using isoelectric focusing (IEF) experiments, and PCR reactions were undertaken to identify ß-lactamase genes in the strains. The OMP profile for each strain was determined and the proteins with decreased expression were submitted to N-terminal sequencing. Four susceptible isolates recovered from the same medical sites at the same period were used as negative controls.

Results: The isolates showed MICs of ≥ 64 mg/L against CTZ, 16-32 mg/L against IMP, 8-16 mg/L against MER. No significant differences in the carbapenems MICs with the inhibitors were observed. IEF experiments showed that the isolates possess 2 to 4 ß-lactamases. These results clustered the isolates in three different groups according to the ß-lactamases profile: (I) isolates with pIs of 9.0, 6.7, 5.8 and 5.4, (II) pIs of 9.0, 6.7, 5.8 and (III) pIs of 9.0, 6.7. One strain of each group was submitted for PCR with customer primers to bla_{TEM}, bla_{CMY}, $bla_{CTX\text{-}M}$, bla_{GES}, bla_{KCP}, bla_{IMIA}, bla_{OXA}, bla_{SHV} ß-lactamase genes. Sequencing analyses of the amplicons confirmed the presence of bla_{TEM}-like.

Conclusions: The decreased susceptibility against carbapenems in Argentinean *Acinetobacter* spp. isolates can be due to decreased expression of porins associated to hyper-expression of ß-lactamases that normally have low affinity to carbapenems. The low levels of *in vitro* resistance against carbapenems may jeopardize the treatment of infections caused by this pathogen.

INTRODUCTION

Acinetobacter spp. has emerged as one of the most important opportunistic pathogens. *Acinetobacter* spp. infections represent an important problem in certain geographic regions, such as Latin America, where this organism is usually more resistant to antimicrobial agents. In recent years, several outbreaks of nosocomial infections caused by carbapenem-resistant *Acinetobacter* spp. have been documented in Latin American countries, especially Argentina and Brazil.

Various mechanisms of ß-lactam resistance have been identified in *Acinetobacter* spp., including: penicillin-binding-protein alterations, reduced uptake of the antimicrobial as a result of loss or reduced expression of outer membrane proteins, and the production of ß-lactamases. Ambler class A ß-lactamases, such as TEM-1, PER-1 and CARB-5, a range of class D oxacillinases, chromosomally encoded cephalosporinase (AmpC), and, less frequently, class B metallo-ß-lactamases such as IMP-1 and VIM-1, have been reported to be associated with ß-lactam resistance in these pathogens.

In this report, we examined 34 carbapenem-resistant clinical isolates of *Acinetobacter* spp. These strains were recovered in Buenos Aires, Argentina and submitted to the SENTRY Antimicobial Surveillance Program in 2002.

MATERIALS AND METHODS

Bacterial isolates. A total of 34 *Acinetobacter* spp. showing resistance to ceftazidime (> 16 mg/L), imipenem (> 8 mg/L) and meropenem (> 8 mg/L) were evaluated. These isolates were recovered during 2002 in Buenos Aires, Argentina and forwarded to JMI Laboratories and the University of Bristol as part of the SENTRY Antimicrobial Surveillance Program.

Susceptibility testing. Expanded antimicrobial susceptibility testing was carried out using agar dilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS). The MICs of ceftazidime, imipenem and meropenem were determined with and without 100 µM BRL 42715, 500 mg/L cloxacillin or 25 mg/L reserpine. In addition, ciprofloxacin was tested with and without reserpine. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

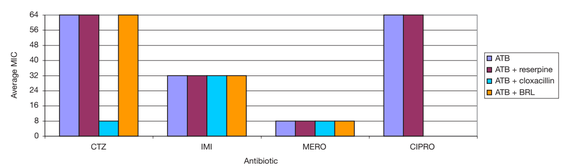
Activity assays. ß-lactamase activity of cell sonicates from overnight broth cultures were determined by spectophotometric assays. These experiments were performed at 299 nm using imipenem and meropenem as substrates.

Analytical IEF. Cell extracts showing ß-lactamase activity were obtained by cell lyses with BugBuster (Novagen, Nottingham, UK). IEF was performed with the NOVEX (Invitrogen, Carlsbad, CA) apparatus. The focused ß-lactamases were detected by overlaying the gel with nitrocefin solution (Microbiology Systems, Cockeysville, MD). Isoelectric points were estimated by linear regression, using the software Graf Prism (GraphPad Softaware Inc., San Diego, CA), by comparison to reference proteins provided by a pI 4.5 to 9.5 Standard IEF Marker (Bio-Rad, Richmond, CA) and the TEM-1 ß-lactamase.

Outer membrane protein (OMP) analysis. OMPs were analyzed from logaritimic phase cultures using SDS-PAGE. The carbapenem-resistant isolates were cultured in 50 ml Nutrient broth with 2 mg/L of imipenem, and purified outer membrane preparations were obtained by treating the cell envelopes with N-lauryl sarcosine. A total of 20 µg/sample of protein was loaded on SDS-PAGE gel.

PCR experiments, DNA sequencing and computer analysis. Molecular screening for bla_{TEM}, bla_{CMY}, $bla_{CTX\text{-}M}$, bla_{GES}, bla_{KCP}, bla_{IMIA}, bla_{OXA}, bla_{SHV} was performed using primers targeted to conserved regions of the ß-lactamase genes. PCR reactions were carried out in standard conditions. The PCR fragments were sequenced on both strands using Perking Elmer systems 377 DNA Sequencer. The nucleotide sequences, deduced amino acid sequences and the phylogenetic relationships were analyzed using Lasergene software package (DNASTAR, Madison, WI). Obtained sequences were compared to sequences available over the internet (http://www.ebi.ac.uk/fasta33/).

FIGURE 1. Effect of the inhibitors reserpine, cloxacillin and BRL42715 (BRL) when associated with ceftazidime (CTZ), imipenem (IMI) and meropenem (MERO), and ciprofloxacin (CIPRO) in the agar dilution tests.



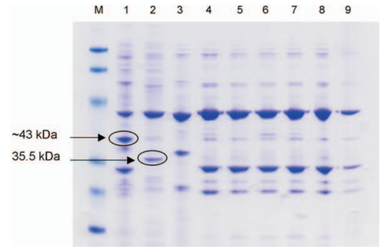
RESULTS

- The isolates showed high level resistance to ceftazidime (MIC, ≥ 64 mg/L) and relatively lower levels of resistance to imipenem (16-32 mg/L) and meropenem (8-16 mg/L).
- There were no significant variations in the MIC results for the ß-lactams when the serine-ß-lactamase inhibitor BRL 42715 or the pump inhibitor reserpine were added (Figure 1).
- A remarkable decrease in the MIC results for ceftazidime was observed when the AmpC inhibitor cloxacillin was added to the susceptibility tests (from ≥ 64 to 8-16 mg/L). However, the carbapenem MIC results did not change (Figure 1).
- Cell extracts showed no detectable hydrolytic activity against carbapenems.
- IEF experiments showed that the isolates possess 2 to 4 ß-lactamases. These results clustered the isolates in three different groups according to the ß-lactamases profile: (I) isolates with pIs of 9.0, 6.7, 5.8 and 5.4, (II) pIs of 9.0, 6.7, 5.8 and (III) pIs of 9.0, 6.7 (Table 1).
- The outer membrane profiles showed decreased expression of OMPs with 35 and 43 kDa (Figure 2).
- PCR and sequencing results showed that $bla_{TEM\text{-}1}$ was present in 28 of 34 (82.3%) isolates evaluated (Table 1).

Table 1. Carbepenem-resistant *Acinetobacter* spp. from Buenos Aires, Argentina grouped according to the analytical IEF profile and results of PCR reactions for $bla_{TEM\text{-}1}$ gene.

				MIC (mg/L)		
	Isolate	pI	PCR $bla_{TEM\text{-}1}$	CAZ	IMI	MER
Group I (16/34)	182	9.0, 6.7, 5.8, 5.4	positive	>64	16	8
	205	9.0, 6.7, 5.8, 5.4	positive	64	16	8
	1409	9.0, 6.7, 5.8, 5.4	positive	>64	16	8
	2073	9.0, 6.7, 5.8, 5.4	positive	>64	32	16
	2078	9.0, 6.7, 5.8, 5.4	positive	>64	16	16
	2673	9.0, 6.7, 5.8, 5.4	positive	>64	16	16
	3848	9.0, 6.7, 5.8, 5.4	positive	>64	16	16
	4034	9.0, 6.7, 5.8, 5.4	positive	>64	16	8
	4282	9.0, 6.7, 5.8, 5.4	positive	>64	16	16
	4463	9.0, 6.7, 5.8, 5.4	positive	>64	16	16
	5001	9.0, 6.7, 5.8, 5.4	positive	>64	32	8
	5837	9.0, 6.7, 5.8, 5.4	positive	>64	16	8
	5842	9.0, 6.7, 5.8, 5.4	positive	>64	16	8
	10569	9.0, 6.7, 5.8, 5.4	positive	>64	16	16
	11703	9.0, 6.7, 5.8, 5.4	positive	>64	16	16
	13622	9.0, 6.7, 5.8, 5.4	positive	>64	16	8
Group II (15/34)	428	9.0, 6.7, 5.8	positive	>64	16	16
	1165	9.0, 6.7, 5.8	negative	>64	32	8
	3456	9.0, 6.7, 5.8	positive	>64	16	8
	3569	9.0, 6.7, 5.8	positive	>64	16	16
	4737	9.0, 6.7, 5.8	positive	>64	16	8
	4742	9.0, 6.7, 5.8	positive	>64	16	8
	4750	9.0, 6.7, 5.8	positive	>64	16	8
	5002	9.0, 6.7, 5.8	positive	>64	32	16
	5204	9.0, 6.7, 5.8	negative	>64	32	16
	5524	9.0, 6.7, 5.8	negative	>64	32	16
	5843	9.0, 6.7, 5.8	negative	>64	16	8
	6490	9.0, 6.7, 5.8	positive	>64	16	16
	7170	9.0, 6.7, 5.8	negative	>64	16	8
	7176	9.0, 6.7, 5.8	positive	>64	16	8
	12370	9.0, 6.7, 5.8	negative	>64	16	8
Group III (3/34)	5004	9.0, 6.7	positive	>64	16	4
	5100	9.0, 6.7	positive	>64	16	16
	5207	9.0, 6.7	positive	>64	16	8

FIGURE 2. Comparison of the outer membrane protein of the *Acinetobacter* spp. from Buenos Aires, Argentina: lanes: (M) molecular weight standards, (1, 2 and 3) susceptible isolates and (4 to 9) resistant isolates. The circles indicate the presence of the 35.5 kDa protein and the 43 kDa protein.



CONCLUSIONS

- Overexpression of AmpC seems to play an important role in the resistance to ceftazidime in the *Acinetobacter* spp. strains evaluated in the present study. However, resistance to the carbapenems was not affected by inhibition of this ß-lactamase.
- Our results show that reduced expression of OMP associated with hyper-expression of ß-lactamases, other than AmpC, contributed to the carbapenem resistance observed in the *Acinetobacter* spp. isolates from Argentina evaluated in the present study.
- Additional studies are still necessary to determine the importance of each resistant determinant in the carbepenems-resistance among *Acinetobacter* spp. strains.

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