BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902017000216111

Article

β-lactamase-producing Gram-negative bacteria in an intensive care unit in southern Brazil

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The present study evaluated the antimicrobial susceptibility profile, β-lactamase production, and genetic diversity of *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. using phenotypic identification, antimicrobial susceptibility testing, and β-lactamase phenotypic detection. Isolates were obtained from patients in an intensive care unit in a hospital in southern Brazil. Bacterial genomic DNA was extracted, followed by the genotypic detection of carbapenemases and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). Fifty-six isolates (26 *Klebsiella pneumoniae*, five *Escherichia coli*, three *Enterobacter aerogenes*, nine *P. aeruginosa*, and 13 *Acinetobacter* spp.) were evaluated. The phenotypic extended spectrum β-lactamase (ESBL) test was positive in 53.8% of the *K. pneumoniae* isolates, 100.0% of the *E. coli* isolates, and 100.0% of the *E. aerogenes* isolates. Phenotypic and genotypic testing of *K. pneumoniae* carbapenemase (KPC) was positive in 50.0% of the *K. pneumoniae* isolates. Phenotypic and genotypic testing showed that none of the *P. aeruginosa* or *Acinetobacter* spp. isolates were positive for metallo-β-lactamase (MBL). The bla_{OXA} gene was detected only in *Acinetobacter* spp. The lowest genetic diversity, determined by ERIC-PCR, was observed among the KPC-producing *K. pneumoniae* isolates and OXA-producing *Acinetobacter* spp. isolates, indicating the inadequate dissemination control of multidrug-resistant bacteria in this hospital environment.

Uniterms: *Enterobacteriaceae*. *Pseudomonas aeruginosa*. *Acinetobacter* spp. Antimicrobial resistance. Beta-lactamases. Bacterial typing. Intensive Care Unit/study/Brazil. Drug-resistant bacteria.

INTRODUCTION

Healthcare-associated infections are among the leading causes of morbidity and mortality in patients and are associated with higher treatment costs (Sydnor, Perl, 2011). Some studies have reported the importance of controlling nosocomial infection to reduce the number of these infections, which provides economic benefits (Gastmeier *et al*., 2006; Rosenthal, Maki, Graves, 2008; Landelle, Marimuthu, Harbarth, 2014).

Pseudomonas aeruginosa, *Acinetobacter* spp., and some members of the *Enterobacteriaceae* family are often involved in nosocomial infections (Mendes *et al*., 2005). For decades, extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria have been an important cause of therapy failure. Carbapenems are usually the only available treatment option (McGowan, 2006). However, carbapenemase-producing Gram-negative pathogens, such as *Klebsiella pneumoniae* carbapenemase (KPC) and metallo-β-lactamase (MBL), are resistant to all available antimicrobial agents, including carbapenems, and have emerged in most hospitals worldwide (Zavascki *et al*., 2009; Nordmann, Naas, Poirel, 2011; Toledo *et al*., 2012; Maragakis, 2010; Cherkaoui *et al*., 2014).

The emergence of multidrug resistance has hampered and limited treatment options. Prompt and adequate treatment is crucial. The management of these life-threatening infections and treatment decisions should be guided by reliable antimicrobial susceptibility testing (Gagliotti *et al*., 2014; Tängdén, Giske, 2015).

The increase in multidrug-resistant pathogens has

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occurred concomitantly with a drastic reduction of the discovery and development of new antimicrobial agents, making these infections more difficult to control (Cassir, Rolain, Brouqui, 2014).

The identification of microorganisms that are involved in nosocomial infections and knowledge of their antimicrobial resistance profiles can guide physicians in choosing the appropriate therapy, playing a key role in epidemiology (Stuart, Leverstein-Van Hall, 2010).

The present study evaluated the antimicrobial susceptibility profile, β-lactamase production, and genetic diversity of *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. isolates that were obtained from patients in an intensive care unit (ICU) in a hospital in southern Brazil from March 2012 to August 2013.

METHODS

Enterobacteriaceae and non-glucose-fermenting Gram-negative (*P. aeruginosa* and *Acinetobacter* spp.) isolates were obtained from patients in an ICU in a hospital in southern Brazil, from March 2012 to August 2013. Only one isolate from each patient was selected and stored at –80 ºC in the Laboratory of Medical Bacteriology, Department of Clinical Analysis and Biomedicine, State University of Maringa. No personal data were taken from patients, the privacy was guaranteed as well the law concerning research with humans (Resolution 466/2012 Brazil National Health Council, Health Ministry). Phenotypic identification and antimicrobial susceptibility testing (AST) were carried out by AUTO-SCAN-4 automated system (Siemens Microscan, Inc., Deerfield, IL, USA). The susceptibility testing was interpreted according to the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2015).

Klebsiella pneumoniae and *Escherichia coli* isolates were tested to ESBL production by confirmatory phenotypic test (CLSI, 2015), and *Enterobacter aerogenes* isolates by disk approximation test (Tumbarello *et al.*, 2004), using cefepime disk (30 mg, Oxoid Basingstoke, England). *K. pneumoniae* ATCC® 700603 and *E. coli* ATCC® 25922 were used as positive and negative controls, respectively.

KPC phenotypic detection was carried out in *Enterobacteriaceae,* which had Minimal Inhibitory Concentration (MIC)≥0.5 μg/mL to meropenem by the modified Hodge test (MHT) (CLSI, 2015). *K. pneumoniae* isolates with and without bla_{KPC} gene, kindly provided by the Central Laboratory of Parana (LACEN) were used as control.

Ceftazidime-resistant *P. aeruginosa* and *Acinetobacter* spp. isolates were evaluated for the presence of MBL by disc-approximation test, using 2-mercaptopropionic acid (2-MPA, Acros, New Jersey, USA) and EDTA (Invitrogen, Carlsbad, USA) (Arakawa *et al*., 2000). IMP-1-producing *A. baumannii* (A-3227) (Gales *et al.,* 2003b) and *P. aeruginosa* ATCC® 27853 were used as positive and negative controls, respectively.

Detection of bla_{KPC}, bla_{OXA}, bla_{SPM}, bla_{IMP}, $bla_{GIM,}$ and bla_{SIM} β-lactamase genes were performed by PCR using Veriti 96-well thermal cycler instrument (Applied Biosystems at Life Technologies, Foster City, CA) and AmpliTaq Gold® PCR master mix (Applied Biosystems at Life Technologies, Hammonton, NJ). The primer sequences and amplicon sizes are shown in Table I.

All isolates were fingerprinted using enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) (Versalovic, Koeuth, Lupski, 1991). Bacterial genomic DNA was extracted by the heat boil method from the overnight bacterial growth (Swanenburg *et al*., 1998). The PCRs were performed using primers ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'), as described by Szczuka and Kaznowski (2004). The

ERIC-PCR fragments obtained were examined by electrophoresis in 2% agarose gels and stained with ethidium bromide. The spectral band analysis on agarose gel was performed using BioNumerics software (version 4.45, Applied Maths, Sint-Martens-Latem, Belgium). The dendrogram was constructed using the Dice coefficient, and the phylogenetic distance was determined using the Unweighted Pair Group Method with Arithmetic Mean algorithm (Sneath, Sokal, 1973). Isolates with ≥ 90% similarity were considered closely related.

RESULTS

The studied institution is a small-size public hospital with 10 adult's ICU beds and pediatric. Fifty-six isolates (26 *K. pneumoniae*, five *E. coli*, three *E. aerogenes*, nine *P. aeruginosa*, and 13 *Acinetobacter* spp.) were tested for susceptibility profile and genotyped.

Resistance profiles for all isolates are shown in Table II. Most of the *Acinetobacter* spp. and *K. pneumonia* isolates were resistance to carbapenems.

Among *Enterobacteriaceae*, the phenotypic ESBL test was positive in 53.8% (14/26) of the *K. pneumonia* isolates, 100.0% (5/5) of the *E. coli* isolates, and 100.0% (3/3) of the *E. aerogenes* isolates. The phenotypic KPC test was positive in 50.0% (13/26) of the *K. pneumoniae* isolates. The KPC enzyme was not detected in the *E. coli* or *E. aerogenes* isolates. Most of the KPC-producing *K.*

TABLE I - Oligonucleotides used in the PCR reactions

Primer[a]	Sequence (5'- 3')	Amplicon size (bp)	Reference
KPC (F) KPC (R)	ATGTCACTGTATCGCCGTCT TTTTCAGAGCCTTACTGCCC	893	Poirel *et al*., 2011
GIM-1(F) GIM-1(R)	AGAACCTTGACCGAACGCAG ACTCATGACTCCTCACGAGC	753	Castanheira *et al*., 2004
IMP-1(F) IMP-1(R)	TGAGCAAGTTATCTGTATTC TTAGTTGCTTGGTTTTGATG	740	Yan *et al*., 2001
SIM-1(F) SIM-1(R)	AGAACCTTGACCGAACGCAG ACTCATGACTCCTCACGAGG	570	Poirel *et al*., 2011
SPM-1(F) SPM-1(R)	CCTACAATCTAACGGCGACC TCGCCGTGTCCAGGTATAAC	650	Gales *et al*., 2003a
OXA-23(F) OXA-23(R)	GATCGGATTGGAGAACCAGA ATTTCTGACCGAATTTCCAT	501	Woodford *et al*., 2006
OXA-24(F) OXA-24(R)	GGTTAGTTGGCCCCCTTAAA AGTTGAGCGAAAAGGGGATT	246	Woodford *et al*., 2006
OXA-51(F) OXA-51(R)	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	353	Woodford *et al*., 2006

[a] F, sense primer; R, antisense primer

TABLE II - Antimicrobial resistance profile of Gram-negative bacteria isolated from patients in an ICU in southern Brazil, from March 2012 to August 2013

Antimicrobials	% resistant isolates				
	K. pneumoniae **(n=26)**	***E. coli*** **(n=5)**	***E. aerogenes*** **(n=3)**	***P. aeruginosa*** **(n=9)**	***Acinetobacter*** **spp. (n=13)**
Cefuroxime	100.0	100.0	100.0	NP	NP
Cefepime	100.0	100.0	100.0	44.3	100.0
Ceftazidime	96.1	100.0	66.6	77.7	84.6
Ciprofloxacin	100.0	60.0	66.6	22.2	100.0
Imipenem	50.0	0.0	0.0	22.2	84.6
Meropenem	50.0	0.0	0.0	22.2	84.6
Ertapenem	73.1	0.0	0.0	NP	NP

NP: Not Performed.

pneumoniae isolates were resistant to all of the antibiotics tested (only 15.4% were sensitive to amikacin). Among the non-KPC-producing *K. pneumoniae* isolates, 46.1% were resistant to ertapenem. All of the KPC-producing *K. pneumoniae* isolates, detected by the MHT test, were positive for the bla_{KPC} gene. None of the other *Enterobacteriaceae* isolates were positive for bla_{KPC}.

None of the ceftazidime-resistant *P. aeruginosa* isolates or *Acinetobacter* spp. isolates were positive for MBL. The bla_{KPC}, bla_{SPM}, bla_{IMP}, bla_{GIM}, and bla_{SIM} genes were not detected in the *P. aeruginosa* or *Acinetobacter* spp. isolates that were resistant to ceftazidime. The bla_{OXA} genes were detected in *Acinetobacter* spp. isolates, in which 100.0% (13/13) were positive for bla_{OXA-51} and 84.6% (11/13) were positive for bla_{OXA-23}.

The ERIC-PCR analysis of 26 *K. pneumoniae* isolates revealed DNA band patterns that could differentiate four isolates, and 84.61% (22/26) of the *K. pneumoniae* isolates formed three clusters, with four, eight, and 10 isolates each. For the five *E. coli* isolates, three were unique, and 2/5 (40%) were clustered. The three *E. aerogenes* isolates presented unique profiles. The nine *P. aeruginosa* isolates could be differentiated into one cluster with four isolates (4/9 [44.44%]) and five isolates with unique patterns. For *Acinetobacter* spp. isolates, 10/13 (76.92%) were clustered, and three had unique patterns.

DISCUSSION

Although sparse official data have been published, bacterial resistance in Brazil is a major challenge to antimicrobial therapy, especially in the southern and southeastern regions (Rossi, 2011; Toledo *et al*., 2012). The present study detected high rates of resistance to third- and fourth-generation cephalosporins and a concerning increase in resistance to carbapenems in Gram-negative bacilli that were isolated from patients in an ICU in a hospital in southern Brazil. In *Enterobacteriaceae*, ESBL production is a decisive mechanism that determines resistance to broad-spectrum cephalosporins. Recent studies by Jones *et al*. (2013), Bonelli, Moreira, and Picão, (2014), and Nogueira *et al*. (2014) corroborate our findings, indicating that this mechanism of resistance is especially common in *K. pneumoniae*, *E. coli*, and *Enterobacter* spp. in Brazil, with important implications for decisions regarding adequate antimicrobial therapy. In the present study, ESBL production was high in *K. pneumoniae*. Because the co-production of KPC prevents interpretations of the results of ESBL phenotypic testing (Poulou *et al*., 2014), we did not test for ESBL genes.

The production of carbapenemases was important for antimicrobial resistance in the *Enterobacteriaceae* and non-glucose-fermenting Gram-negative isolates. The predominant carbapenemases include New Delhi metallo-β-lactamases (NDM), Oxacillinases (OXA), Verona integron-encoded metallo-β-lactamase (VIM), Imipenemase (IMP), and KPC, which are encoded by the genes bla_{NDM}, bla_{OXA}, bla_{VIM}, bla_{IMP}, and bla_{KPC}, respectively (Nordman, Naas, Poirel, 2011). In Brazil, some types of carbapenemases have been detected more frequently, such as São Paulo metallo-β-lactamase (SPM) in *P. aeruginosa* isolates (Rossi, 2011; Rizek *et al*, 2014.), OXA-23 in *Acinetobacter* spp. isolates, and KPC in *Enterobacteriaceae* isolates (Rossi, 2011, Biberg *et al*., 2015; Rieger *et al*., 2016). The identification of carbepenemase production is currently the most successful way by which carbapenem resistance is determined among members of the *Enterobacteriaceae* family (Gupta *et al*., 2011). In the present study, the bla_{KPC} gene was detected in 50% of the *K. pneumoniae* isolates, and bla_{OXA} was detected in all of the *Acinetobacter* spp. isolates. The MBL genes (bla_{SPM}, bla_{IMP}, bla_{GIM}, and bla_{SIM}) were not detected in any of the isolates tested.

The phenotypic MHT test presented high sensitivity in detecting KPC-producing *K. pneumonia* in the present study, which was confirmed by genotypic testing. Raghunathan, Samuel, and Tibbetts (2011) reported similar results, with sensitivity as high as 90%. However, Girlich, Poirel, and Nordmann (2012) found that the phenotypic MHT test had low sensitivity in detecting other carbapenemases, particularly MBL, such as NDM.

The presence of MBL is quite common in *P. aeruginosa*. However, despite the significant resistance of the isolates to carbapenems, this enzyme was not detected by our phenotypic test. Genotypic testing, which targets the most common bacterial MBL genes that are associated with resistance, was negative. These results suggest other mechanisms of resistance, such as the direct actions of efflux pumps or the loss of porins.

The $bla_{\text{OXA-51}}$ gene was detected in all of the *Acinetobacter* spp. isolates, which may indicate that all of them belonged to the *A. baumannii* species, which naturally harbors this gene in its chromosome (Héritier *et al*., 2005). The OXA-51 family, similar to β-lactamases, comprises several members and has weak activity against carbapenems. When these enzymes are expressed *in vivo*, however, they can increase the MIC of carbapenems, leading to a resistant isolate. Although few studies have implicated OXA-51 in the resistance to carbapenems, these enzymes are nonetheless concerning and have been shown to confer resistance to carbapenems in *A. baumannii* (Evan, Amyes, 2014). Furthermore, over 80% of the *Acinetobacter* spp. isolates that were resistant to carbapenems were also positive for $bla_{\text{OXA-23}}$. The $bla_{\text{OXA-23}}$ gene is able to confer resistance to carbapenems, although high levels of resistance (MIC≥32 μg/mL) were observed only when $bla_{\text{OXA-23}}$ was associated with other mechanisms (Evan, Amyes, 2014).

ERIC-PCR revealed high genetic similarity among KPC-producing *K. pneumoniae*. This finding raises concerns about inadequate control in the studied hospital environment and the dissemination of multidrug-resistant bacteria. KPC-producing *Enterobacteriaceae* was first reported in Brazil in 2005 and has spread, becoming endemic in many hospitals (Bonelli, Moreira, Picão, 2014). A previous study in southern Brazil was based on a surveillance program for multidrug-resistant bacteria and found that KPC-producing *Enterobacteriaceae* increased from 17% in 2010 to over 80% in 2011 (Toledo *et al*., 2012). Of the KPC-producing *K. pneumoniae* and OXA-producing *Acinetobacter* spp., 12/13 (92.3%) and 10/11 (91.0%), respectively, belonged to the same cluster. This sharp increase in such a short period of time emphasizes the importance of this kind of antimicrobial resistance in Brazil.

CONCLUSION

High antimicrobial resistance to carbapenems, mainly by β-lactamase-producing *Enterobacteriaceae*

and non-glucose-fermenting Gram-negative, was detected in isolates from patients in an ICU in southern Brazil. The genetic similarities among the KPC-producing *K. pneumoniae* isolates and among the OXA-producing *Acinetobacter* spp. should alert health professionals to the necessity of implementing suitable measures for infection control in this studied environment.

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Received for publication on 16th June 2016
Accepted for publication on 15th February 2017