Molecular characterization of *Klebsiella pneumoniae* carbapenemase-producing isolates in southern Brazil

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Carbapenem-resistant *Enterobacteriaceae* have been frequently reported worldwide. They represent a serious concern because of the limited therapeutic options. The aim of this study was to investigate the molecular epidemiology of 14 *Klebsiella pneumoniae* carbapenemase (KPC) producers among 345 clinical isolates of *Enterobacteriaceae* with reduced susceptibility to carbapenems recovered from 11 separate hospitals in southern Brazil. The *bla*<sub>KPC-2</sub> gene was detected in 14 isolates (4 %): six *Enterobacter cloacae*, five *K. pneumoniae* and three *Serratia marcescens*. Most of these isolates exhibited high-level resistance against β-lactams and ciprofloxacin, while the most active drugs were polymyxin B and amikacin. Genetic environment analysis, based on the classical Tn<sub>4401</sub> structure, revealed six distinct platforms. Plasmids carrying *bla*<sub>KPC-2</sub> were not typable and most were approximately 20 kb. Only KPC carbapenemases were found among the isolates studied, highlighting the local relevance of these enzymes in acquired resistance to carbapenems in *Enterobacteriaceae*. Our results contribute to the understanding of carbapenem resistance in *Enterobacteriaceae* and to the molecular characterization of KPC-2-producing isolates in Brazil.

INTRODUCTION

Carbapenem-resistant *Enterobacteriaceae* have been frequently reported worldwide (Brink *et al.*, 2012; Gales *et al.*, 2012; Gupta *et al.*, 2011) and severely limit treatment options (Magiorakos *et al.*, 2012; Nordmann *et al.*, 2009). Although a large diversity of carbapenemases is found among *Enterobacteriaceae*, one of the major enzymes responsible for carbapenem resistance in this family is *Klebsiella pneumoniae* carbapenemase (KPC) (Giakkoupi *et al.*, 2011; Gupta *et al.*, 2011). KPCs are plasmid-mediated enzymes that are disseminated worldwide and currently represent an important public health problem (Walsh, 2010). KPC producers have been increasingly detected in Brazil since 2009 (Andrade *et al.*, 2011; Monteiro *et al.*, 2009; Pavez *et al.*, 2009; Pereira *et al.*, 2013; Zavascki *et al.*, 2009). The aim of this study was to investigate the molecular epidemiology of KPC producers recovered from 11 distinct hospitals in southern Brazil.

METHODS

**Bacterial isolates.** A total of 345 non-duplicate clinical isolates resistant or with reduced susceptibility to carbapenems, with a zone of inhibition of ≤21 mm to ertapenem (ERT) and/or meropenem (MEM) (CLSI, 2009), were selected from June 2009 to July 2011 as part of a regional surveillance project to detect carbapenem resistance among *Enterobacteriaceae*. The isolates were recovered from 11 hospitals in southern Brazil. These isolates had been previously identified by conventional techniques in the institutions of origin. The automated Vitek 2 system (bioMérieux) was used to confirm the identification of the KPC-producing isolates. The susceptibility
profile was initially evaluated by disk diffusion. The MICs for carbapenems were determined for all isolates using broth microdilution and interpreted according to Clinical and Laboratory Standards Institute breakpoints (CLSI, 2012). MICs for ceftazidime, cefepime, ampicillin-sulbactam, amikacin, ciprofloxacin, polymyxin B and tigecycline were evaluated only against KPC-producing isolates. Susceptibility to polymyxin B was interpreted according to CLSI breakpoints for *Pseudomonas aeruginosa* (CLSI, 2012) and for tigecycline according to European Committee on Antimicrobial Susceptibility Testing breakpoints (EUCAST, 2013).

**β-Lactamase-encoding genes and clonal relatedness.**

Carbapenemases were investigated in all isolates by multiplex real-time PCR for detection of *bla*KPC, *bla*VIM, *bla*GES, *bla*NDM, *bla*OXA-48 and *bla*IMP genes (Monteiro et al., 2012). Conventional PCRs were performed to detect the presence of *bla*ESBL-1 carbapenemase and *bla*CTX-M extended-spectrum β-lactamase genes (Gaspareto et al., 2007; Paterson et al., 2003). Sequencing of the *bla*KPC gene was performed as previously described (Yigit et al., 2001). PCR products were purified using the ExoStar kit (GE Healthcare) and sequenced using a BigDye Terminator kit (version 3.1) and an ABI 3500 Genetic Analyzer (Applied Biosystems). GenBank was used to access the KPC sequences deposited to date and the BioEdit program was used to compare similarities between sequences. The positive control strains included KPC-producing *K. pneumoniae* American Type Culture Collection (ATCC) BAA-1705 and the VIM-, IMP-, SPM-, GES-, NDM- and OXA-48-producing isolates referred to by Carvalhaes et al. (2013).

Clonal relatedness among KPC producers was established by DNA macrorestriction using the *XbaI* enzyme, followed by pulsed-field gel electrophoresis (PFGE) using the CHEF-DR II apparatus (Bio-Rad). The results were analysed using BioNumerics software (Applied Maths) and similarity >85% upon dendrogram analysis was considered to represent the same PFGE pattern groups.

**Genetic environment and plasmid characterization of KPC-producing isolates.** The genetic environment of *bla*KPC was investigated by amplification of the Tn4401 sequence as described by Curiao et al. (2010) with addition of the primers: istB-f 5'-GC-TGGACCATGCTCTATCTTGCT-3' and KPC-r2 5'-CGTAACGGATGGTGTCCTCA-3'; KPC-r2 5'-GACTGGCGAGTCTGAGA-3' and tnpA-(r) 5'-CGTGAAAGATGCAAGGTCAATG-3' (Fig. 1). The non-conserved region between *istB* and *bla*KPC was analysed by comparison of the RFLP patterns obtained by digestion of amplicons with BamHI (Andrade et al., 2011).

Plasmid analysis using PCR-based replicon typing (PBRT) was performed to detect replicons of the major plasmid incompatibility (Inc) groups (Carattoli et al., 2005). The identification of plasmids was accomplished using genomic DNA digested by *SI* enzyme followed by PFGE and hybridization with specific probes.

**RESULTS**

Among the 345 *Enterobacteriaceae* isolates, 224 (64.9 %) were identified as *Enterobacter* spp. (75 % *Enterobacter cloacae*, 2.7 % *Enterobacter aerogenes* and 22.3 % identified only to genus level), followed by 94 (27.2 %) *Klebsiella* spp. (98.9 % *K. pneumoniae* and 1.1 % *K. oxytoca*), 18 (5.2 %)

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**Fig. 1.** Schematic representation showing (a) intact classical Tn4401 and (b) the modified genetic environment found in the bacteria studied. *Fragments amplified by PCR; **performed with RFLP. Dashed lines indicate no amplification. Primers in black indicate the amplification strategies proposed by Curiao et al. (2010) and primers *istB*-f, KPC-r2 and tnpA-(r) indicate the additional strategies described in this study. *Kp*, *K. pneumoniae*; *Sm*, *S. marcescens*; *Ecl*, *Enterobacter cloacae*.
Escherichia coli, eight (2.3%) Serratia marcescens and one (0.3%) Proteus mirabilis. The isolates were recovered from several clinical specimens, including urine, blood, sputum, venous catheter and surgical wounds.

The \textit{bla}_{KPC} gene was identified in 14 (4.1%) isolates from three of the 11 hospitals: six (42.9%) \textit{Enterobacter cloacae}, five (35.7%) \textit{K. pneumoniae} and three (21.4%) \textit{S. marcescens}. Sequencing of \textit{bla}_{KPC} revealed 100% identity with the \textit{kpc2} gene. None of the other carbapenemase genes investigated was detected among the isolates. High-level resistance against most \beta-lactam drugs was observed among KPC-2-producing isolates; amikacin and polymyxin B presented the highest susceptibility rates (Table 1). Co-production of CTX-M was detected in two isolates of \textit{K. pneumoniae} (22PRO and 134PRO) belonging to the same pulso group K2. PFGE analysis showed two different PFGE pattern groups for KPC-producing \textit{Enterobacter cloacae} (E1 and E2), with 79.4% similarity. The isolates belonging to E1 and E2 clones presented 100% identity. Among the KPC-producing \textit{K. pneumoniae}, four distinct PFGE pattern groups were observed (K1– K4). The two isolates classified as pulso group K2 showed genomic similarity of 86.7%. Two PFGE pattern groups were identified for KPC-producing \textit{S. marcescens} (S1 and S2). Isolates belonging to the S1 group were 100% identical, whereas the S2 group presented a similarity of 82.4% to S1 (Table 1).

Analysis of the genetic environment of \textit{bla}_{KPC-2} genes showed six distinct platforms (i–vi) compared with the classical Tn4401, as shown in Fig. 1. All isolates showed the presence of the insertion sequence (IS) \textit{Kpn6} (\textit{tnpA}) just downstream to \textit{bla}_{KPC-2}. On the other hand, IS\textit{Kpn7} (\textit{istA} and \textit{istB}) was only detected in \textit{K. pneumoniae} 134PRO, whereas partial amplification of this IS was accomplished for both \textit{S. marcescens} 177HC and 79PRO, and for \textit{K. pneumoniae} 144PRO. \textit{K. pneumoniae} 134PRO also presented a fragment of 200 bp in the variable region between \textit{istB} and \textit{bla}_{KPC}. The Tn3 (\textit{tnpR} and \textit{tnpA}) upstream to IS\textit{Kpn7}–\textit{bla}_{KPC-2}–IS\textit{Kpn6} was detected in \textit{K. pneumoniae} 22PRO and 144PRO and \textit{S. marcescens} 151PRO. The study of the inverted repeats (right and left, IRR and IRL) was not totally successful (Fig. 1).

Plasmid analysis revealed that plasmids carrying \textit{bla}_{KPC-2} were approximately 20 kb in most of the isolates (Table 1). Only \textit{Enterobacter cloacae} 189HC and \textit{K. pneumoniae} 134PRO and 144PRO (pulso groups K3 and K4, respectively) harboured \textit{bla}_{KPC-2} in different plasmid sizes, respectively approximately 100, 48 and 30 kb. Although different Inc groups were observed among the isolates, all plasmids associated with \textit{bla}_{KPC-2} were untypable, according to the PBRT scheme.

A total of 331 isolates (95.9%) were identified as non-carbapenemase producers. The distribution of carbapenem MICS demonstrated distinct patterns according to each bacterial genus (Table 2). Independently of the level of resistance to ERT, most \textit{Enterobacter} spp. remained susceptible to other carbapenems. On the other hand, for \textit{Klebsiella} spp., susceptibility to imipenem (IPM) and MEM was considerably reduced among isolates that were highly resistant to ERT.

**DISCUSSION**

KPC production in \textit{Enterobacteriaceae} is an increasing problem worldwide (Gupta et al., 2011). In Brazil, the first evidence of KPC-producing \textit{Enterobacteriaceae} was from isolates recovered in 2005 (Pavez et al., 2009; Zavascki et al., 2010). However, outbreaks have been reported in several Brazilian regions since 2009, with endemic levels of KPC-2-producing isolates noted in many regions (Andrade et al., 2011; Pereira et al., 2013). Despite the increasing incidence of these organisms in our country, only sporadic cases have been detected in Rio Grande do Sul, the southernmost Brazilian region (Andrade et al., 2011; Ribeiro et al., 2012; Zavascki et al., 2009).

In this study, we evaluated isolates resistant or with reduced susceptibility to carbapenems and investigated carbapenemase-encoding genes detected only the presence of \textit{bla}_{KPC-2} among the population analysed. A low prevalence (4%) of KPC-2-producing isolates was found when compared with other Brazilian regions, which are facing an increasing incidence of this carbapenemase (Andrade et al., 2011; Pereira et al., 2013). High resistance rates to \beta-lactams and high ‘in vitro’ susceptibility to amikacin were observed in our study, with a similar profile to other KPC-2-producing isolates recovered in Brazil and other countries (Andrade et al., 2011; Hirsch & Tam, 2010). According to PFGE analysis, two distinct PFGE pattern groups were observed among \textit{Enterobacter cloacae} and \textit{S. marcescens}. The co-production of a CTX-M by \textit{K. pneumoniae} 22PRO probably contributed to the higher MICs for ceftazidime (16-fold) compared with those obtained for the other K2 clone (149PRO).

Tn4401, a Tn3-based transposon, is the genetic environmental structure that generally contains \textit{bla}_{KPC} genes (Cuzon et al., 2010). The classic isoforms ‘a’ and ‘b’ have been reported in Brazil in different genera of \textit{Enterobacteriaceae} (Andrade et al., 2011; Pereira et al., 2013). We found six distinct platforms for the isolates studied here, compared with the classical Tn4401, confirming a considerable diversity of structures surrounding \textit{bla}_{KPC}, as already shown by several authors (Chen et al., 2012; Gootz et al., 2009; Pereira et al., 2013). The unsuccessful amplification of IR sequences in most of the isolates suggests a distinct insertion site or deletions. It should be noted that the absence of amplification for IRR and IRL has also been reported for other KPC producers in Brazil (Pereira et al., 2013). Only one isolate (134PRO) presented a variable region compatible with the isoform ‘b’ of Tn4401, but the absence of amplification of \textit{tnpR} and \textit{tnpA} makes interpretation difficult, considering that this region characterizes the inherited structure of Tn3 transposon. For some other isolates only a partial amplification of IS\textit{Kpn7} was observed, possibly indicating the
Table 1. Clinical, laboratory and molecular data for KPC-2-producing isolates

Resistance is indicated in bold and underlining indicates the size of the plasmid-carrying \textit{bla}_{KPC-2}.

\textit{ND}, Not detected; AMI, amikacin; AMS, ampicillin-sulbactam; CAZ, ceftazidime; CIP, ciprofloxacin; CPM, cefepime; IPM, imipenem; PB, polymyxin B; TGC, tigecycline; Tn, transposon.

<table>
<thead>
<tr>
<th>KPC-producer</th>
<th>Hospital</th>
<th>MIC ((\mu g/ml))</th>
<th></th>
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<th></th>
<th></th>
<th>PFGE pattern group</th>
<th>Plasmid analysis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IPM</td>
<td>MEM</td>
<td>ERT</td>
<td>CPM</td>
<td>CAZ</td>
<td>AMS</td>
<td>AMI</td>
<td>CIP</td>
</tr>
<tr>
<td><em>Enterobacter cloacae 5HC</em>†</td>
<td>A</td>
<td>64</td>
<td>128</td>
<td>256</td>
<td>512</td>
<td>128</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<td>A</td>
<td>128</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;512</td>
<td>256</td>
<td>&gt;256</td>
<td>&gt;512</td>
<td>&gt;64</td>
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<td>&gt;256</td>
<td>&gt;512</td>
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<tr>
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<td>16</td>
<td>&gt;256</td>
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<td>64</td>
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</tr>
<tr>
<td><em>K. pneumoniae 174HC</em></td>
<td>A</td>
<td>256</td>
<td>128</td>
<td>128</td>
<td>64</td>
<td>32</td>
<td>&gt;256</td>
<td>4</td>
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<tr>
<td><em>K. pneumoniae 22PRO§</em></td>
<td>B</td>
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<td>&gt;512</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>&gt;64</td>
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<tr>
<td><em>K. pneumoniae 134PRO</em></td>
<td>C</td>
<td>32</td>
<td>32</td>
<td>128</td>
<td>512</td>
<td>16</td>
<td>&gt;256</td>
<td>4</td>
<td>&gt;64</td>
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<tr>
<td><em>K. pneumoniae 144PRO</em></td>
<td>A</td>
<td>8</td>
<td>32</td>
<td>128</td>
<td>16</td>
<td>64</td>
<td>&gt;256</td>
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<tr>
<td><em>K. pneumoniae 149PRO§</em></td>
<td>B</td>
<td>4</td>
<td>4</td>
<td>16</td>
<td>32</td>
<td>16</td>
<td>&gt;256</td>
<td>16</td>
<td>2</td>
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<tr>
<td><em>S. marcescens 177HC</em></td>
<td>A</td>
<td>16</td>
<td>8</td>
<td>32</td>
<td>32</td>
<td>256</td>
<td>&gt;256</td>
<td>2</td>
<td>&gt;64</td>
</tr>
<tr>
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<td>128</td>
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</tr>
<tr>
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<td>&lt;1</td>
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</tr>
</tbody>
</table>

*Plasmids (Inc groups) detected in this study did not carry \textit{bla}_{KPC-2} genes.
†Platforms established according to Fig. 1.
‡Plasmids carrying \textit{bla}_{KPC-2} genes of these isolates were originally investigated and reported by Andrade et al. (2011).
§CTX-M producers.
occurrence of another transposition event, as previously reported (Cuzon et al., 2010; Pereira et al., 2013). Furthermore, the fact that most of the isolates had no amplification between \textit{istB} and the \textit{bla\textsubscript{KPC}} gene could be a determinant of the high-level resistance to carbapenems observed among these KPC-2 producers. Previous reports have shown that deletions in this variable region (directly upstream to the \textit{bla\textsubscript{KPC}} gene) can affect gene expression and influence the level of carbapenem resistance (Gootz et al., 2009; Kitchel et al., 2010). In hospital A, the platform ‘vi’ was found in most of the \textit{Enterobacter cloacae} isolates, regardless of the clonal profile. The same platform was also identified among two isolates of \textit{K. pneumoniae} belonging to distinct clones and from different institutions. On the other hand, isolates belonging to the same clonal group presented different platforms, such as \textit{Enterobacter cloacae} 189HC (E1 clone; platform ‘vi’) and \textit{K. pneumoniae} 22PRO (platform ‘ii’) and 149PRO (platform ‘vi’), both from K2 clones. These results show that there is no clear relationship between the genetic environment and the institutions nor with the clonal profile of the isolates. Overall, our results confirm that there is a considerable diversity of structures surrounding the \textit{bla\textsubscript{KPC}-2} gene.

All KPC producers except three had \textit{bla\textsubscript{KPC}} found on plasmids of approximately 20 kb in size, and none of the \textit{bla\textsubscript{KPC}}-containing plasmids were typable according to the PBRT classification system. A large diversity of plasmids of several sizes and distinct Inc groups have been associated with KPC-producing isolates around the world (Andrade et al., 2011; Chen et al., 2013; Pereira et al., 2013; Ribeiro et al., 2012), highlighting the successful dissemination of this genetic resistance determinant.

In this study, most isolates with reduced susceptibility to ERT were confirmed to be non-carbapenemase producers and presented high susceptibility to IPM and MEM. This profile is probably a result of the action of weaker $\beta$-lactamases (extended-spectrum $\beta$-lactamase or AmpC) coupled with porin loss, as already well demonstrated by several authors. Our results reinforce the finding that ERT is a poor indicator of carbapenemase presence (Doumith et al., 2009).

Only KPC carbapenemases were detected among isolates in this study, highlighting the local relevance of these enzymes in acquired resistance to carbapenems. Our results contribute to the understanding of carbapenem resistance in \textit{Enterobacteriaceae} and to the molecular characterization of KPC-2-producing isolates in Brazil.

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