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# Characterization and molecular epidemiology of extensively prevalent nosocomial isolates of drug-resistant *Klebsiella pneumoniae*

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Genet. Mol. Res. 18 (1): gmr18172
Received October 08, 2018
Accepted February 01, 2019
Published February 25, 2019
DOI http://dx.doi.org/10.4238/gmr18172

**ABSTRACT.** We characterized six drug-resistant nosocomial isolates of *Klebsiella pneumoniae* obtained in a hospital located in northern Minas Gerais State, Brazil, by determining their antibiotic sensitivity profiles, detecting the $bla_{KPC}$ genetic marker and examining their clonal relationships. All isolates were found to be extensively drug resistant. A PCR assay was used to confirm the identity of the isolates as *K. pneumoniae* and assess the $bla_{KPC}$ gene. All isolates tested positive for the $bla_{KPC}$ gene, which is related to carbapenem resistance. The genetic profiles and clonal relationships among the isolates were evaluated by ERIC-PCR. All the isolates were in a single group with two distinct subgroups. Analysis of the

 

genetic diversity among the isolates revealed that five of the six were clones, which suggests cross-transmission in this hospital environment. Five of the patients died from infection. We describe the first detection of KPC-producing *K. pneumoniae* isolates from a hospital in northern Minas Gerais state.

**Key words:** *Klebsiella pneumoniae*; Nosocomial infections; $bla_{KPC}$ gene; ERIC-PCR

## INTRODUCTION

*Klebsiella pneumoniae* is an opportunistic human pathogen that causes numerous healthcare-associated infections (HAIs), including urinary tract infections, post-surgical infections, infections of the lungs and soft tissue, and bacteremias (Hou et al., 2015; Shen et al., 2016; Zhan et al., 2017). Treatment of these HAIs is a major challenge, as they are typically associated with a high mortality rate of 40 – 50% (Borer et al., 2009; Liang et al., 2017). Selective pressure, exerted by the extensive use of antibiotics, has led to the emergence of multidrug-resistant strains of *K. pneumoniae* that are resistant to several antibiotic classes, including carbapenems. In addition, genetic transmission of resistance genes on mobile elements has promoted the transfer of resistance among species and genera, aggravating the problem of antibiotic resistance in *K. pneumoniae* (Hou et al., 2015).

The emergence and spread of carbapenem-resistant Enterobacteriaceae poses a significant threat to public health. Among the determinants of carbapenem resistance in *K. pneumoniae* are a group of variant carbapenem-degrading enzymes. The main mechanism of carbapenem resistance in *K. pneumoniae* is carbapenemase production. The strains of *K. pneumoniae* that hydrolyze imipenem and/or meropenem are classified as class A, B, or D, according to genetic differences (Ambler) or as 2f, 3a, or 3b according to their substrate preference and molecular structure (Bush-Jacoby-Medeiros) (Yang et al., 2013; Djahmi et al., 2014; Abbas and Jarallah, 2017).

A variety of Ambler molecular class A carbapenemases have been described; some are encoded on a chromosome (NMC, SME, and IMI), while others are encoded on a plasmid (KPC and GES) (Diene and Rolain, 2014; Abbas and Jarallah, 2017). KPC carbapenemases are the most frequent Ambler molecular class A enzymes (Djahmi et al., 2014). These enzymes hydrolyze most β-lactams and have the greatest potential for dissemination; in part because they are located on plasmids, but mainly because they are so frequently found in *K. pneumoniae*, which is notorious for its ability to accumulate and transfer resistance determinants. A carbapenemase of the KPC family was first reported in a clinical isolate of *K. pneumoniae* in North Carolina in 1996 (Yigit et al., 2001; Queenan and Bush, 2007; Djahmi et al., 2014). So far, there are 24 known variants of KPC, and KPC-2 and KPC-3 are the most frequent worldwide (Peirano et al., 2009; Maya et al., 2013; Djahmi et al., 2014; Yu et al., 2016; Chakraborty, 2016; Araújo et al., 2018). In Brazil, the first carbapenemase KPC-type was reported in 2006 (Maya et al., 2013). *Klebsiella pneumnoniae* KPC-2 was found in clinical isolates recovered from two hospitals in Rio de Janeiro, Brazil (Peirano et al., 2009). Recently *Klebsiella pneumnonia* KPC-2 has also been isolated from nosocomial samples from several Brazilian regions (Araújo et al., 2018).

 

We looked for $bla_{KPC}$ in nosocomial carbapenem-resistant isolates of *K. pneumoniae* obtained from patients in a hospital in northern Minas Gerais State and investigated the clonal relationships among these strains, based on their molecular epidemiological profiles.

## MATERIAL AND METHODS

### Bacterial isolates and antimicrobial susceptibility profiles

This study was performed in a hospital in southeast Brazil that is categorized as a general tertiary-level hospital, with 377 beds. From January–June 2016, six non-duplicate clinical carbapenem-resistant isolates (one isolate per patient) of *K. pneumoniae* were obtained. The isolates were obtained from blood, urine, and tracheal aspirate samples from patients in various sectors (intensive care unit, apartments, and wards) of the hospital. The isolates were identified at the genus level by biochemical and enzymatic tests in the clinical laboratory of the hospital based on standard microbiology identification methods. Susceptibility to several classes of antibiotics was determined by the disc diffusion method according to CLSI guidelines (CLSI, 2017). *Klebsiella pneumoniae* isolates that were resistant to imipenem and meropenem were stored in Brain Heart Infusion medium (BHI; BD®) containing 20% glycerol at -20°C.

### DNA extraction and PCR for the bacterial 16S rDNA and *K. pneumoniae* 16S rDNA genes

The cryopreserved isolates were reactivated by incubation in BHI medium (Laborclin®) at 37°C. After 24 h of growth, the microbial suspensions were spread on 90-mm blood agar plates (Laborclin®) and incubated for 24 h. Isolated colonies picked from the plates were subjected to DNA extraction with the KAPA Express Extract kit (Kappa Biosystems) according to the manufacturer's protocol. DNA was eluted in a final volume of 100 µL and then quantified by 1% agarose gel electrophoresis and used for PCR and ERIC-PCR. Identification of the bacterial isolates as *K. pneumoniae* was confirmed by PCR using universal bacterial 16S rDNA primers and *K. pneumoniae* 16S rDNA primers generating amplicons of 370 bp and 130 bp, respectively (Greisen et al., 1994; Al-Jalawi et al., 2014). Primers described by Yigit et al. (2001) to screen for the $bla_{KPC}$ gene; they generated an 876 bp fragment. All primers were synthesized by Integrated DNA Technology (USA). The amplifications were performed in a single PCR containing 2× GoTaq Green Master Mix® (Promega, USA), 2.5 mM $MgCl_2$, 10 µM of each primer, and 50 ng of bacterial DNA in a final volume of 50 µL. The amplification conditions used were the same as those reported by the authors listed for each primer. The amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide and photographed. As a positive control for the PCR, a nosocomial strain of *K. pneumoniae* previously identified by a Brazilian reference laboratory (Ezequiel Dias Foundation), encoding the bacterial 16S rDNA gene and $bla_{KPC}$, was used. Distilled and sterilized water was used as a negative control.

## Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and statistical analysis

The genetic profiles and clonal relationships among the *K. pneumoniae* isolates were evaluated by polymorphism analysis of genomic DNA.

For this purpose, two primers, ERIC-1 5′-TGTAAGCTCCTGGGGATTAAC-3′ and ERIC-2 5′-AAGTAAGTGACTGGGGTGAGCG-3′, were used in a repetitive intergenic enterobacterial consensus PCR assay, as described previously. The amplification was performed in a single PCR containing 2× GoTaq Green Master Mix, 2.5 mM $MgCl_2$, 10 µM of each primer, and 50 ng of bacterial DNA in a final volume of 50 µL. The amplification conditions were as described by Duan et al. (2016). The amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide and photographed. The amplification profiles were visually analyzed by two observers, and their observations were transformed into binary data in a matrix, according to the presence (1) or absence (0) of bands. To evaluate the genetic relationships among the isolates, the matrix was subjected to cluster analysis by the nearest neighbor method (UPGMA) based on the Jaccard coefficient for automatic generation of the dendrogram using the MultiVariate Statistical Package (MVSP) version 3.12 h (GeoMem, Blairgowrie, UK). As a positive control, a nosocomial strain of *K. pneumoniae* previously identified by a Brazilian reference laboratory (Ezequiel Dias Foundation) encoding the $bla_{KPC}$ gene was used. As a negative control, *Staphylococcus aureus* strain ATCC 43300 was used.

## Accession and analysis of patient records

After obtaining authorization from the hospital, and in accordance with ethical guidelines, the medical records for the six patients from which the multidrug-resistant *K. pneumoniae* strains were isolated were accessed and analyzed. The data sought were the illness related to the patient's hospitalization, comorbidities, age, sex, date of hospitalization, date of isolation of the bacteria, hospitalization sector, and duration of hospitalization.

## Ethical approval

This study was approved by the research ethics committee of the participating hospital as well as the research ethics committee of the State University of Montes Claros, Minas Gerais, Brazil (protocol no. 855.002/2014).

# RESULTS

## Epidemiological profiles of the patients and their *K. pneumoniae* isolates

The isolates were obtained from a variety of sources, including blood (n = 2), tracheal aspirates (n = 1), and urine (n = 3). The resistance profiles of all six *K.*

 

*pneumoniae* isolates analyzed in this study showed that they were extensively drug resistant.

Epidemiological analysis of the six patients from whom the carbapenem-resistant *K. pneumoniae* strains were isolated revealed that the mean age was 57 years (SD = 16.6; range, 35–72 years); three patients were ≥61 years old, one was between 51 and 61 years old, and two were between 30 and 50 years old. The illnesses related to the patients' hospitalization were hematological or solid neoplasms (2), chronic renal failure (2), chronic obstructive pulmonary disease (1), and pulmonary thromboembolism (1). The mean number of days elapsed between hospital admission and bacterial isolation was 52 days. Regarding the hospitalization unit, two were in apartments, three were in wards, and one was in an intensive care unit. The mortality rate due to infection with multidrug-resistant *K. pneumoniae* is known to be high; in our study, the mortality rate was five of the six cases.

## Antimicrobial susceptibility profiles of the isolates and PCR amplification and sequencing of the bacterial universal 16S rRNA, *Klebsiella pneumoniae* 16S rRNA and bla$_{KPC}$ genes

The antimicrobial susceptibility testing of the *K. pneumoniae* isolates revealed a high degree of resistance to the drugs; thus, they were considered to be extensively drug-resistant (XDR) (Table 1) (Magiorakos et al., 2012).

**Table 1.** Antimicrobial resistance profiles, species confirmation by amplification of the universal bacterial and *Klebsiella pneumoniae*-specific 16S rDNA genes, and screening for the carbapenem-resistance *bla*$_{KPC}$ gene.

| Isolate | Date of isolation | Hospital section | Antimicrobial resistance profile | Universal Bacterial 16S rDNA gene | *Klebsiella pneumoniae* 16S rDNA gene | *Bla*$_{KPC}$ gene | Genetic profile by ERIC-PCR | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Group | Genotype |
| ISO 1 | 03/26/2016 | Nursing ward | ACC, AMP, ASB, AZT, CFZ, CFP, CFS, CAZ, CRO, CFX, CIP, IPM, LVX, MER, PPT, SXT. | + | + | + | II | IIB |
| ISO 2 | 05/18/2016 | ICU | ACC, AMP, ASB, AZT, CFZ, CFP, CFS, CAZ, CRO, CFX, CIP, IPM, LVX, MER, PPT, SXT. | + | + | + | II | IIA |
| ISO 3 | 05/14/2016 | Nursing ward | ACC, AMP, ASB, AZT, CFZ, CFP, CFS, CAZ, CRO, CFX, CIP, IPM, LVX, MER, PPT, SXT. | + | + | + | II | IIA |
| ISO 4 | 05/23/2016 | Apartment | ACC, AMP, ASB, AZT, CFZ, CFP, CFS, CAZ, CRO, CFX, CIP, IPM, LVX, MER, PPT, SXT. | + | + | + | II | IIA |
| ISO 5 | 06/15/2016 | Apartment | ACC, AMP, ASB, AZT, CFZ, CFP, CFS, CAZ, CRO, CFX, CIP, IPM, LVX, MER, PPT, SXT. | + | + | + | II | IIA |
| ISO 6 | 06/29/2016 | Nursing ward | ACC, AMP, ASB, AZT, CFZ, CFP, CFS, CAZ, CRO, CFX, CIP, IPM, LVX, MER, PPT, SXT. | + | + | + | II | IIA |

Abbreviations: ANX, nalidixic acid; AMC, amikacin; ACC, amoxicillin + clavulanic acid; AMP, ampicillin; ASB, ampicillin + sulbactam; AZT, Aztreonam; CFZ, cefazolin; CFP, cefepime; CFS = cefoxitin sodium; CAZ, ceftazidime; CRO, ceftriaxone; CFX, cefuroxime; CIP, ciprofloxacin; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MER, meropenem; NTA, nitrofurantoin; PPT, piperacillin + tazobactam; SXT, sulfamethoxazole-trimethoprim; and ICU, intensive care unit.

All isolates in this study (n = 6) showed a 370-bp fragment corresponding to the bacterial 16S rRNA gene (Figure 1A) and a 130-bp fragment confirming that the species was *K. pneumoniae* (Figure 1B). For all six isolates (100%) the PCR amplified an expected fragment of 876 bp, corresponding to the $bla_{KPC}$ gene (Figure 1C and Table 1).

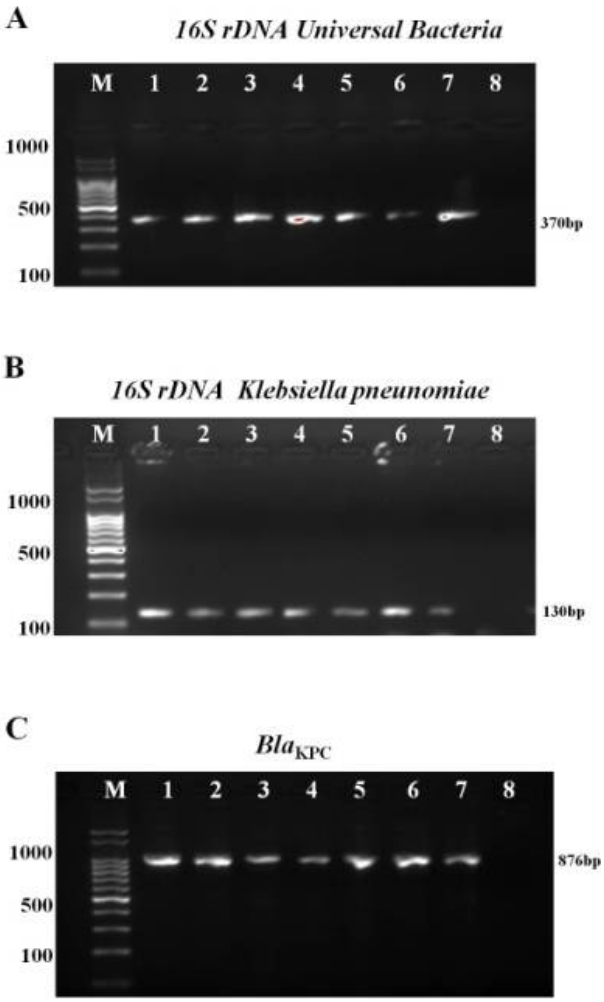


**Figure 1.** Characterization of six nosocomial carbapenem-resistant isolates of *K. pneumoniae* by PCR amplification of the universal bacterial 16S rDNA gene, *Klebsiella pneumoniae* 16S rDNA gene and *blaKPC* gene related to carbapenem-resistance. Shown are three 1.5% agarose gels containing amplicons. M: 100 bp molecular weight marker (Ludwig). Panel A: PCR for the detection of the universal bacterial 16S rDNA gene. Lanes 1–6: isolates 1–6. Lane 7: positive control (a genotyped *K. pneumoniae* strain). Lane 8: negative control (no DNA). Panel B: PCR for *K. pneumoniae* 16S rDNA gene. Lanes 1–6: isolates 1–6. Lane 7: positive control (a genotyped *K. pneumoniae* strain). Lane 8: negative control (no DNA). Panel C: PCR for detection of *blaKPC*. Lanes 1–6: isolates 1–6. Lane 7: positive control (a genotyped *K. pneumoniae* strain). Lane 8: negative control (no DNA). The expected sizes of the fragments corresponding to the universal bacterial 16S rDNA gene, *K. pneumoniae* 16S rDNA gene and *blaKPC* gene are shown in the respective panels.

 

## Analysis of the genetic profiles and clonal relationships among the carbapenem-resistant *K. pneumoniae* isolates

Genotypic analysis of the six *K. pneumoniae* isolates using ERIC-PCR produced 14 fragments of different sizes (Figure 2). For the band sharing analysis, bands in the range of 100–2080 bp were considered. The six isolates were grouped into a single group (II) and two subgroups (IIA and IIB), with two distinct genotypes (Figure 3 and Table 1).

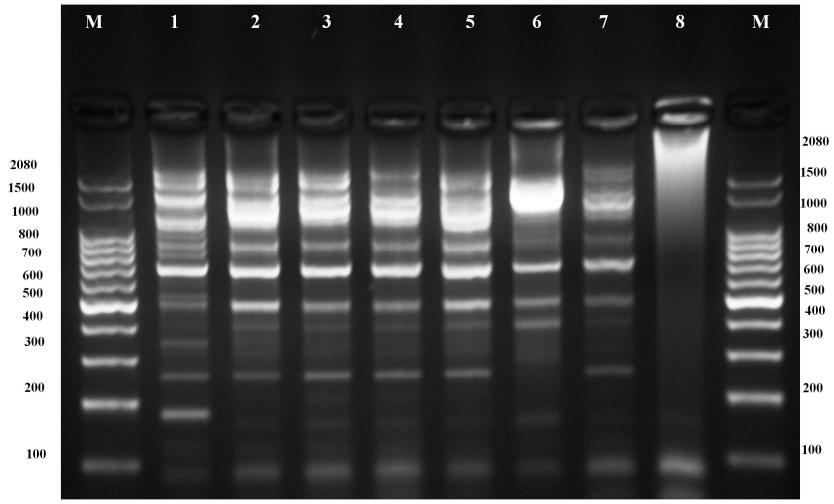


**Figure 2.** ERIC-PCR to determine the genetic profile of nosocomial carbapenem-resistant *Klebsiella pneumoniae* isolates. Shown is a 1.5% agarose gel containing the following M: molecular weight marker, 100 base pair DNA ladder (Ludwig). Lanes 1–6: nosocomial *K. pneumoniae* isolates 1– 6. (ISO 01–06). Lane 7: positive control (a genotyped *K. pneumoniae* strain). Lane 8: negative control (*Staphylococcus aureus* strain, ATCC 43300). The sizes of the bands in the 100 base pair DNA marker are shown on the left and right of the gel.

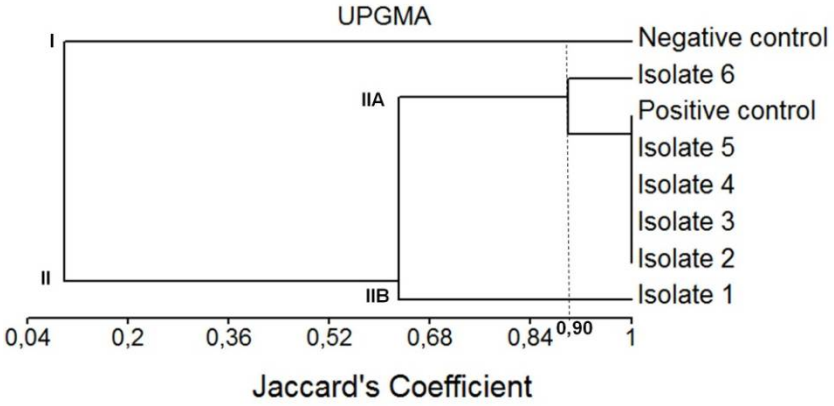


**Figure 3**. Dendrogram of the genetic relationships among the nosocomial carbapenem-resistant *Klebsiella pneumonia* isolates obtained by ERIC-PCR. Isolates 1–6 were distributed in one group (II) and two subgroups (IIA and IIB) according to the degree of similarity.

We considered strains with a Jaccard coefficient ≥0.9 as identical, and five of the isolates had identical profiles, suggesting clonality. Combined analysis of the antimicrobial resistance profiles, genotypes, and data regarding the sector where the patients were hospitalized and the date of sample collection revealed that the same strain of *K. pneumoniae* was present in various patients (IIB clones) at the same time point in neighboring locations within the hospital. These data suggest the possibility of cross-transmission of the bacteria from one patient to another. Of the five patients infected with this genotype (IIB), four died.

One isolate (IIA) had a Jaccard coefficient of 0.65 when compared to the other isolates (IIB). The band analysis showed the presence or absence of three bands, which led us to the hypothesis of a possible mutation (Figures 2 and 3).

## DISCUSSION

A high prevalence of drug resistance microorganisms has been reported. In a study by Djuric et al. (2016), more than 95% of the analyzed strains were multidrug resistant, and 65% had an extended spectrum beta-lactamase. All *K. pneumonie* nosocomial isolates in our study were drug resistant (Table 1). The mean age of patients from whom the carbapenem-resistant *K. pneumoniae* strains in this study was comparable to the mean age of the patients in similar studies, reporting 60 years (Seibert et al., 2014) and 61 years (Souza et al., 2016). Three of the six patients were male; in studies by Seibert et al. (2014) and Souza et al. (2016), the majority of patients were male. The mortality rate found in this our study was high five of six patients. In a study by Pereira et al. (2016) the mortality rate was 81%; in a meta-analysis conducted by Xu et al. (2017), the mortality rate was 42%; and in a study by Seibert et al. (2014), the mortality rate was 44%. Xu et al. (2017) reported that the high mortality rate observed in patients with multidrug-resistant *K. pneumoniae* is related to their overall health and physical conditions.

The antimicrobial susceptibility profiles of the *K. pneumoniae* isolates in our study showed that all of them were resistant to IPM and MER drugs. In a study by Dellacorte et al. (2016), low sensitivity of *K. pneumoniae* isolates to IPM and MER was also observed. In a study by Seibert et al. (2014), among the tested enterobacterial isolates, *K. pneumoniae* showed greater resistance to carbapenems. In a study by Yan et al. (2017), 37.2 and 30.8% of the isolates were resistant to IPM and MER, respectively.

In recent years, the widespread use of carbapenems has accelerated the spread of carbapenem-resistant strains worldwide (Hawser et al., 2011). Yan et al. (2017) reported that increased use of carbapenems may result in significant selective pressure, promoting the progression of resistance. Resistance to carbapenems in *Klebsiella* spp. is most commonly related to the production of class B β-lactamases and less frequently to the production of class D β-lactamases.

In our study, all isolates amplified an expected fragment corresponding to the $bla_{KPC}$ gene (Figure 1C and Table 1) indicating the possibility that the resistance to the carbapenems imipenem and meropenem was due to this gene.

In general terms, our ERIC PCR results show that the major carbapenen resistant *K. pneumoniae* isolates had identical profiles. Three sectors of the hospital, the ICU, wards, and apartments, with very distinctive characteristics in terms of both the types of patients and the physical structures, were the probable sites of acquisition/transmission of the

 

bacterium. Similar results, including the genetic profiles of the patients, the place and time when they were hospitalized, were observed in a previous study of nosocomial *A. baumannii* isolates from the same hospital (Carvalho et al., 2016; Cheikh et al., 2018).

Local epidemiology data is important for antimicrobial target choice due to the high rates of microbial resistance oagainst carbapenems in Brazil and worldwide. Considering the diversity of resistance genes and their easy dissemination in *K. pneumoniae*, studies of the local epidemiology of *Klebsiella* spp. are very important for providing optimal treatment, epidemiological control, and for preventing the dissemination of these microorganisms within hospitals or even between hospitals (Lee et al., 2017). In addition, the rapid detection of patients colonized or infected with KPC-containing strains is very important since these microorganisms can cause serious infections that have high morbidity and mortality rates (Yan et al., 2017). ERIC-PCR is a fast, inexpensive method that gives good results for the genetic characterization of *Klebsiella* spp (Dalmolin et al., 2017). Genetic techniques are increasingly being used for the identification of microorganisms; one of these techniques is complete genome sequencing. Roach et al. (2015) cited this technique as useful for clinical microbiology; it has recently been used in several epidemiological and molecular studies (Roach et al., 2015; Zhang et al., 2016).

## CONCLUSIONS

We found a high prevalence of $bla_{KPC}$ among nosocomial XDR *Klebsiella* spp. isolates in a north of Minas Gerais hospital. ERIC-PCR analysis was used to evaluate the genetic diversity among the isolates, and although there were some heterogeneous profiles, most showed 100% clonality. These data, suggesting cross-transmission and likely environmental colonization in some hospital care units, indicate the importance of adopting preventive measures at various levels and the need to review current measures to help prevent HAIs, including contact precautions and patient isolation to reduce the chances of cross-transmission, physical area and clinical practices that adhere to the best practices for hospital infection control; identification of the sources of germs, and evaluation of the methods used to clean the environment. The high resistance of the isolates to the major antimicrobials in our study suggests selective pressure and their possible indiscriminate use. Therefore, the adoption of measures aimed toward the rational use of these drugs is critical. Important elements for control should include differentiation between colonization, and infection, limiting treatment to infections and knowledge of the antimicrobial sensitivity profiles of these microorganisms.

## FUNDING

Research supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig).

## CONFLICTS OF INTEREST

The authors report no conflicts of interest

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