

Original Article

Molecular characterization of carbapenem-resistant *Klebsiella pneumoniae* isolates from a university hospital in Brazil

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Abstract

Introduction: The emergence of *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (KPC-Kpn) isolates is attracting significant attention in nosocomial infection settings. *K. pneumoniae* is the main pathogen that harbours *bla*_{KPC} genes.

Methodology: This study evaluated 54 *K. pneumoniae* carbapenem-resistant isolates from patients hospitalized at the University Hospital of Londrina, between July 2009 and July 2010. The isolates were phenotypically screened for carbapenemase production and submitted for genotypic confirmation by polymerase chain reaction (PCR) for KPC, metallo- β -lactamases, OXA-48, and extended-spectrum beta-lactamase genes. The absence of outer membrane proteins (OMP) was investigated by SDS-PAGE. The susceptibility profile was determined by broth microdilution, according to Clinical and Laboratory Standards Institute protocol.

Results: All isolates were phenotypically positive for class A carbapenemase production, but negative for metallo- β -lactamase activity. PCR analysis demonstrated that all isolates carried *bla*_{KPC} genes and sequencing showed that all strains belonged to KPC-2 subtype. Four strains did not show porin expression, and all isolates were resistant to ertapenem, meropenem, and imipenem. Susceptibility rates reached 35.2% for gentamicin, 85.2% for polymyxin B, 87% for colistin, and 98.1% for both tigecycline and fosfomycin. Pulsed-field gel electrophoresis showed six clones, and three of them predominated among the isolates.

Conclusions: KPC-2-producing *K. pneumoniae* is becoming predominant among carbapenem-resistant *K. pneumoniae* isolates at the hospital. The association of the enzyme KPC with other resistance determinants, such as loss of porins, may increase the severity of the situation of nosocomial infections. There is an urgent need to develop strategies for infection control and prevention.

Key words: *Klebsiella pneumoniae*; carbapenemase; KPC; carbapenem resistance.

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Introduction

Klebsiella pneumoniae is an important pathogen that can cause urinary tract infections (UTIs), bacteremia, and pneumonia, especially in patients with significant underlying disease [1,2]. It can contain extended-spectrum beta-lactamase (ESBL) genes, which are associated with multidrug resistance. For this reason, the treatment must be empirical, usually with a carbapenem, while awaiting culture data [2]. According to Correa *et al.*, the available effective antibiotic options are becoming insufficient [3]. Currently, resistance to carbapenems is being increasingly detected worldwide. This is worrying, because this drug class is the chosen

one for the treatment of serious infections caused by multidrug-resistant isolates [4]. As reported by Kantopolou *et al.*, polymyxins could be used as an alternative last choice to treat infections due to resistant Gram-negative bacteria [5].

The production of carbapenemase enzymes is the main mechanism that confers carbapenem resistance in *K. pneumoniae* [3]. The most common carbapenemases in *K. pneumoniae* are the *K. pneumoniae* carbapenemases (KPC) [6]. KPC-associated infections are predominantly nosocomial, involving patients with multiple risk factors, and the mortality rates can be high [7]. However, not only carbapenemases

production/expression can determine carbapenem resistance in isolates of *K. pneumoniae*. These isolates can also present ESBL or AmpC-type β -lactamase genotype. Outer membrane protein (OMP) production, also called porins, can also be altered, becoming an important contributor to carbapenem resistance [8,9]. These proteins are capable of forming water channels, which allow the product trade between the cell and the outer environment [10]. When the bacterial cell loses these channels or stops expressing the encoding genes for it, there is a reduction of the entry of antibiotics into the cell, decreasing the internal concentration of the antimicrobial agent. These events can culminate in resistance to the β -lactams [11].

To block the advance of infections caused by resistant microorganisms, the epidemiology of the mechanisms behind them should be studied; for that, distinction between carbapenem-resistance mediated by carbapenemases and resistance mediated by other mechanisms is important [12]. Carbapenemase-encoding genes are often found in plasmids. The transfer of carbapenemase-encoding genes between strains and different species represents the most difficult challenge in the infection control field [13].

According to data from the University Hospital of Londrina State University (HU-UDEL), *K. pneumoniae* showed carbapenem resistance (Kpn-CR) in 2009. Of the tested *K. pneumoniae* isolates, 10.2% showed carbapenem resistance, and that figure climbed to 55.6% in 2010, mainly as result of dissemination of the *bla*_{KPC} genes (Eliana C. Vespero, personal communication). This scenario became a constant concern at the hospital, and it attracted our attention to the importance of this research. After the first outbreak, many cases of infection and colonization by KPC enzymes occurred, culminating in a second outbreak in August 2010 (Eliana C. Vespero, personal communication). Within this context, our study aimed to evaluate microbiological, epidemiological, and molecular aspects of Kpn-CR strains isolated from hospitalized patients during the period between July 2009 and July 2010.

Methodology

Bacterial strain selection

Clinical isolates of *K. pneumoniae* with decreased susceptibility to carbapenems (ertapenem minimum inhibitory concentration [MIC] ≥ 0.5 mg/L, or imipenem or meropenem MIC ≥ 1 mg/L) identified in the microbiology laboratory at HU-UDEL between July 2009 and July 2010 were selected. Only one isolate per patient and only those with characterized infection

according to the Centers for Disease Control and Prevention (CDC) criteria were studied and submitted for phenotypic testing [14]. Bacterial identification and the initial antibiotic susceptibility testing were performed using the Microscan Walkaway automated system (Siemens, Sacramento, USA).

Phenotypic testing

The isolates were screened for carbapenemase production using the modified Hodge test (MHT), according to Lee *et al.*, and the aminophenylboronic acid test (APB) for the class A β -lactamase producing phenotype, according to Tsakris *et al.* [15,16]. The phenotypic test to detect MBLs was performed with ethylenediamine tetra-acetic acid (EDTA) at 100 mM, according to Picão *et al.* [17].

Antibiotic susceptibility testing

The broth microdilution method (BMD) was performed for antimicrobial susceptibility testing. The following antibiotics were used: imipenem (MSD, Kenilworth, USA), meropenem (ABL, São Paulo, Brazil), ertapenem (MSD, Kenilworth, USA), tigecycline (Wyeth, Dallas, USA), colistin (USP, Rockville, USA), polymyxin B (Sigma-Aldrich, St. Louis, USA), and gentamicin (Sigma-Aldrich, St. Louis, USA). BMD with cation-adjusted Muller-Hinton broth (Sigma-Aldrich, St. Louis, USA) was carried out in accordance with Clinical Laboratory Standards Institute (CLSI) document M7-A8 [18]. Bacterial suspensions were adjusted according to CLSI recommendations, and the final inoculum was verified for BMD susceptibility methods. Each strain's susceptibility to fosfomycin (Sigma-Aldrich, St. Louis, USA) was tested by the agar dilution method, according to CLSI 2011 guidelines [19]. For tigecycline and colistin, the breakpoints used were from the US Food and Drug Administration (FDA) and European Committee on Antibiotic Susceptibility Testing (EUCAST), respectively [20]. The American Type Culture Collection (ATCC) strains used for test validation were as follows: *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853, *Staphylococcus aureus* 29213, *Enterococcus faecalis* 29212, *K. pneumoniae* 700603, and *K. pneumoniae* 13883.

Molecular analysis

Polymerase chain reaction (PCR) was used to screen the strains for the *bla*_{KPC} gene, as described by Bradford *et al.* [21]. The presence of MBL genes (IMP-1, -2, -4, -7, -12; VIM-1, -2, -7; SIM-1; GIM-1; and SPM-1) was investigated by multiplex PCR, as

described by Ellington *et al.* [22]. For identification of OXA-48, the tests were performed according to Poirel [23]. SHV, TEM, and CTX-M groups 1, 2, 8, 9, and 26 genes were also investigated, according to Woodford *et al.* [24]. The KPC amplicons were quantified by spectrophotometry, using Nanodrop (Nanodrop Technologies INC, Wilmington, USA), and were Sanger sequenced using MegaBACE 1000 (ABI 3730 DNA Analyser; Applied Biosystems, Alameda, USA).

Outer membrane protein analysis

To search for other resistance determinants, SDS-Page was also performed to investigate absence of outer membrane proteins (OMPs). The porin extraction followed the protocol of Pavez, and the analysis by SDS-Page was performed according to Laemmli [25,26].

Macrorestriction analysis

Pulsed-field gel electrophoresis (PFGE) was utilized to perform an analysis of the chromosomal DNA [27]. Chromosomal DNA was digested with *XbaI* (Invitrogen, Carlsbad, USA), and the restricted DNA fragments were separated using the CHEF-DRIII system (Bio-Rad, Hemel Hempstead, UK), with pulses ranging from an initial 5-second pulse to a final pulse of 60 seconds, with a voltage of 6 V·cm⁻¹, at 14°C for 23 hours. The λ Lambda Ladder PFG Marker (New England Biolabs, Ipswich, UK) was used as a molecular marker in the gel (50–1,000 kb). The products were stained by ethidium bromide (Invitrogen, Carlsbad, USA) and observed under UV light. The sizes of the fragments were first normalized according to the molecular weight of the DNA markers, and then the fingerprints were analyzed using BioNumerics software (Applied Mathematics, Kortrijk, Belgium, version 4.6), with a position tolerance of 3%. The samples were submitted to similarity analyses using the UPGMA algorithm (unweighted pair-group method, with

arithmetic mean) with the Dice coefficient. DNA fragments greater than 12,000 bp or smaller than 200 bp were excluded from the analysis.

Retrospective review of patients' data

The medical records of the 54 patients were analyzed after the tests, and some risk factors were considered relevant for KPC acquisition and infection. The life habits, comorbidities, hospital unit, and underlying diseases were some points taken for analysis. Data was tabulated in Microsoft Access 2010 and analyzed with SPSS version 19.0 (IBM, Armonk, USA). For nominal variables, the Chi-square test with Yates correction or Fisher's exact test were used. To compare means in independent groups, the Mann-Whitney test was chosen. P values lower than 0.05 were considered statistically significant.

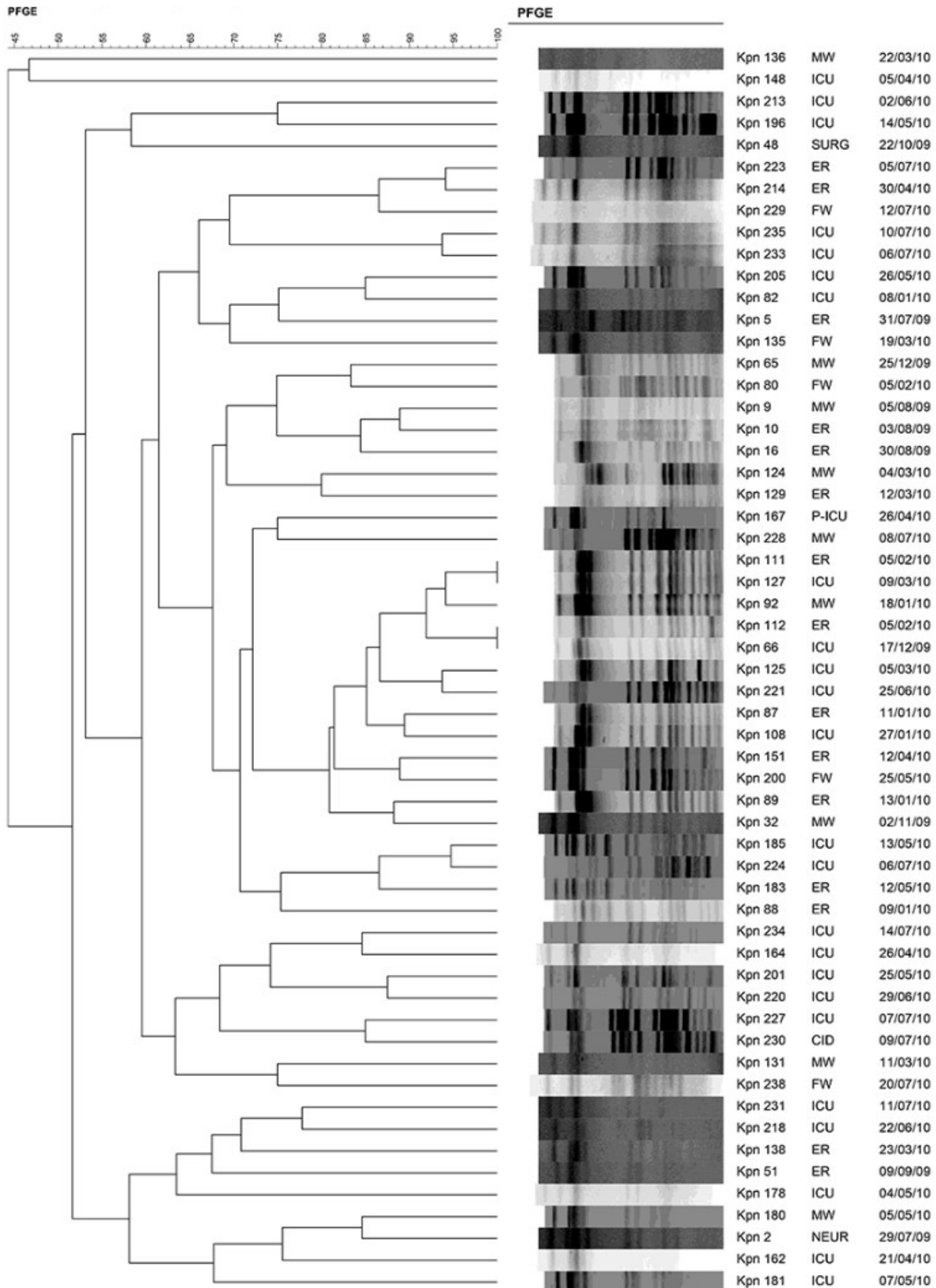
Results

In the studied period, 325 carbapenem-resistant *K. pneumoniae* strains were detected in patients at the hospital. One isolate per patient was included in this study, resulting in 54 samples from different patients, each of which caused infection according to CDC criteria [14]. The bacterial isolates were collected mainly from patients diagnosed with infections of the urinary tract (35.2%), lower respiratory tract (35.2%), bloodstream and central venous catheter (14.8%), skin and soft tissue (11.1%), and abdomen or peritoneum (3.7%). All isolates were positive for MHT and APB tests and negative for EDTA test. When the isolates were evaluated by broth microdilution, all of them were resistant to ertapenem, meropenem, and imipenem. Among the isolates, 35.2% remained susceptible to gentamicin, 85.2% to polymyxin, 87% to colistin, and 98.1% to tigecycline. Only one strain showed resistance to fosfomicin, by agar dilution, corresponding to 98.1% susceptibility.

Table 1. Resistance profile of the 54 *Klebsiella pneumoniae* isolates: combinations of resistance determinants (A–F: different clones).

| Resistance profile | No. of isolates | A | B | C | D | E | F |
|--|-----------------|---|---|---|---|---|---|
| KPC-2 | 6 | 3 | 2 | - | 1 | - | - |
| KPC-2 / porin loss or decreased expression | 1 | - | 1 | - | - | - | - |
| KPC-2 / CTX-M | 2 | - | 2 | - | - | - | - |
| KPC-2 / TEM / SHV | 1 | - | - | - | - | - | 1 |
| KPC-2 / TEM / CTX-M | 7 | 1 | 4 | - | - | - | 2 |
| KPC-2 / SHV / CTX-M | 17 | 8 | 7 | 1 | - | 1 | - |
| KPC-2 / TEM / SHV / CTX-M | 17 | 4 | 8 | - | - | - | 5 |
| KPC-2 / CTX-M / porin loss or decreased expression | 1 | - | 1 | - | - | - | - |
| KPC-2 / CTX-M / TEM / porin loss or decreased expression | 1 | - | - | 1 | - | - | - |
| KPC-2 / CTX-M / SHV / porin loss or decreased expression | 1 | 1 | - | - | - | - | - |

Figure 1. Clonal distribution by PFGE presented in dendrogram by Jaccard similarity coefficient, with date and isolation unit.



Kpn: *Klebsiella pneumoniae*; MW: Men ward; ICU: Intensive Care Unit; SURG: Surgery; ER: Emergency Room; FW: Female Ward; P-ICU: Pediatric Intensive Care Unit; CID: Center of Infectious Diseases; NEUR: Neurology.

The PCR results showed that all isolates were *bla*_{KPC} carriers, and sequencing confirmed that all of them belonged to the *bla*_{KPC-2} subtype. Multiplex PCR did not detect any metallo-β- lactamase or OXA-48 genes. According to the results of PCR for ESBL genes, eight strains did not show ESBL genes. The CTX-M-positive samples had different profiles, presenting different combinations of genes. Four samples lacked OmpK 36 porin, three of them combined with CTX-M genes (2 isolates: porin loss and CTX-M 2, 8, and 26; 1 isolate: porin loss and CTX-M 1) (Table 1).

The different clonal types were designated A–F (Figure 1). The PFGE analysis of the 54 KPC-Kpn isolates identified one major clone (B), which comprised 25 (46.3%) isolates and included subtypes B1 (n = 10), B2 (n = 1), B3 (n = 2), B4 (n = 4), B5 (n = 6), and B6 (n = 2). This clone appeared in August of 2009 and remained at a high frequency for most of the following months. A second prevalent clone (A), with 17 (31.5%) isolates, included subtypes A1 (n = 6), A2 (n = 4), A3 (n = 1), A4 (n = 3), A5 (n = 2), and A6 (n =

1). This was the first clone to appear after the outbreak and was detected almost every month until July of 2010. Clones A and B showed 75% genetic similarity between them. Eight isolates (15%) were clonal type F, including subtypes F1 (n = 3), F2 (n = 1), F3 (n = 2), and F4 (n = 2), which are 70% genetically similar with clones A and B. Two clones with 1 (1.9%) isolate each were designated as clonal types E and D, and clone C comprised 2 isolates (3.8%). The medical records of the 54 patients infected with KPC-Kpn were retrospectively reviewed (Table 2). Follow-up was possible until the patients either died or were discharged from the hospital. The patients’ age, gender, underlying diseases, and antibiotic use were recorded for analysis. Previous antibiotic treatment was a clinical characteristic found to increase the likelihood of infection by KPC-Kpn in this study. All patients were treated with multiple antimicrobial agents – often simultaneously (*i.e.*, carbapenem, piperacillin-tazobactam, colistin, third- or fourth-generation cephalosporins, and/or fluorquinolones) – before the

Table 2. Bivariate analysis of demographic and clinical characteristics of patients with infections caused by Carbapenem Resistant *Enterobacteriaceae*.

| Characteristic | Non-survivors (n = 34) | Survivors (n = 20) | P |
|---|---------------------------|-----------------------|--------|
| Age (years), median (range) | 61.2 (12–91) | 47.1 (0.75–77) | 0.011 |
| Male gender, n (%) | 23 (63.8) | 14 (63.6) | 0.719 |
| Comorbidities > 2 | 20 (55.5) | 3 (13.6) | > 0.99 |
| Risk factors, n (%) | | | |
| Mechanic ventilation | 28 (77.7) | 3 (13.6) | |
| Diabetes | 10 (27.7) | 7 (31.8) | 0.905 |
| Heart failure | 11 (30.5) | 3 (13.6) | > 0.99 |
| COPD | 3 (8.3) | 1 (4.5) | > 0.99 |
| Kidney failure | 6 (16.6) | 2 (9) | 0.459 |
| Etilism | 4 (11.1) | 4 (18.2) | 0.462 |
| Tabagism | 6 (16.6) | 1 (4.5) | 0.235 |
| HIV | --- | 1 (4.5) | 0.379 |
| Hepatitis | 2 (5.5) | 1 (4.5) | > 0.99 |
| Cancer | 6 (16.6) | 1 (4.5) | 0.235 |
| SAH | 15 (41.6) | 12 (54.5) | 0.787 |
| Stroke | 3 (8.3) | 1 (4.5) | 0.392 |
| Antibiotic therapy during the last month before KPC-2 <i>K. pneumoniae</i> Isolation | | | |
| Colistin | 11 (30.5) | 2 (9) | 0.040 |
| Carbapenems | 21 (58.3) | 4 (18.2) | 0.001 |
| Penicilins | 5 (13.9) | 3 (13.6) | 0.718 |
| Vancomycin | 14 (38.9) | 7 (31.8) | 0.498 |
| Third-generation cephalosporins | 6 (16.6) | 2 (9) | 0.698 |
| Fourth-generation cephalosporins | 6 (16.6) | 2 (9) | > 0.99 |
| Macrolides | 6 (16.6) | 0 | 0.145 |
| Piperacillin-tazobactam | 10 (27.7) | 5 (22.7) | 0.342 |
| Tigecycline | 4 (11.1) | 2 (9) | 0.697 |
| Fluoroquinolones | 9 (25) | 2 (9) | 0.332 |
| Aminoglycosides | 3 (8.3) | 1 (4.5) | > 0.99 |
| Sulfamethoxazole-trimetoprim | 3 (8.3) | 1 (4.5) | - |

COPD: chronic obstructive pulmonary disease; SAH: Systemic arterial hypertension.

isolation of KPC-Kpn infections. The patients who had previously been treated with carbapenems and colistin had higher mortality rates.

Discussion

According to an estimate from CDC, *K. pneumoniae* can be responsible for 8% of nosocomial infections [28]. Landman reported that more than one-third of Kpn-CR isolates can possess *bla*_{KPC} genes, especially in endemic areas [29]. In this context, the spread of KPC-producing *K. pneumoniae* is of great concern [30-33]. In the present study, we analyzed 54 Kpn-CR isolates collected in HU-UEL, between July 2009 and July 2010, to determine the number of β -lactamases that were expressed in the isolates and the genomic relatedness of them using PFGE.

Our results agree with several Brazilian authors who have demonstrated the endemicity of KPC among isolates of Enterobacteriaceae, especially *K. pneumoniae* [34,35]. The detection of KPC producers is important to understand the epidemiology of hospital infections and could be useful in planning intervention in preventable factors that can reduce the spread of resistance in a hospital setting. However, laboratory detection can be difficult, because routine laboratories usually do not possess molecular diagnostic resources [36]. In this study, MHT and APB showed to be a specific and sensitive phenotypic test for detection of KPC enzymes in regions with great frequency of isolates producing carbapenem resistance.

The results show the high frequency of KPC in our hospital, mainly associated with the ESBL CTX-M, corresponding to 85% of the isolates. A wide variety of CTX-M was found in this study, but 50% belonged to CTX-M group 2. Our results reinforce Yang *et al.*'s report that claims that carbapenemases and porin loss combined with CTXM-2 ESBL can be the main mechanisms behind resistance to carbapenems in *K. pneumoniae* [37]. Among the 54 isolates, only 4 presented loss or had lower expression of both major porins, all associated with KPC production. One of the isolates with KPC and CTX-M 2, 8, and 26 genes that lost porin expression had MICs higher than 128 for colistin and polymyxin B. The other strain with this profile presented higher MICs for carbapenems (> 128). The other isolates without *OmpK 36* expression did not show a significant rise of MIC.

Some authors [38,39] have shown that some isolates with MICs for imipenem/meropenem within the susceptibility range can possess KPC enzymes, and then the detection of the carbapenemases may be difficult. In this study, all isolates were resistant to

carbapenems, showing a MIC₉₀ of 64 μ g/mL for meropenem/imipenem and a MIC₉₀ >128 μ g/mL for ertapenem. Fosfomycin and tigecycline were the most effective antimicrobial agents, according to MIC tests. The strains showed high susceptibility to colistin, polymyxin, tigecycline and fosfomycin, making these therapeutic options for the treatment of KPC-Kpn infections. Given the limited number of remaining therapies, it is crucial to control the spread of this resistance mechanism.

Previous studies have shown that the spread of KPC occurs person to person as a result of breaking the infection control chain [38,40,41]. The results of this molecular epidemiological study show the presence of three major clones, with clone B being the most prevalent. Many authors [42,43] have described similar results, concluding that the dissemination of KPC enzymes requires transmissible plasmids. This concordance suggests that resistant isolates are being selected through antibiotic use. In this study, a larger proportion of deaths were not associated with any specific clone; however, clones A and B showed mortality rates of approximately 60%, and clone F showed a mortality rate of approximately 75%. All of the patients with infections caused by strains of clones C and E died. This study also contained seven patients infected with strains resistant to colistin and polymyxin B; all of these patients died. Four isolates from this cohort belonged to clone B, two belonged to clone A, and one belonged to clone F.

The concentration of positive KPC-Kpn cases in the intensive care unit (ICU) and in the emergency room (ER) is likely due to the invasive procedures (such as mechanical ventilation) with which the patients of these units are treated. For this reason, some patients from other wards could have been contaminated with KPC when passing through one of these contaminated areas (ICU and ER), as most of the patients spent time in one or both of these wards at some point during their hospitalization. Due to their resistance to most available antimicrobial agents, invasive infections by these organisms have been associated with high rates of morbidity and mortality. Patients who are hospitalized for prolonged periods of time and those with severe underlying disease are at risk of acquiring these types of pathogens.

Conclusions

Carbapenemase KPC-2 is emerging, which is associated with significant mortality. KPC-2-producing *K. pneumoniae* is becoming endemic in isolates of Kpn-CR in the hospital, and its association with other

resistance determinants can increase the threat. In concordance with Garcia-Sureda *et al.*, the multidrug resistance phenotype results from the progressive accumulation of different mechanisms of resistance in the same microorganism, including high-level production of ESBL and porin deficiency [44]. Our findings highlight the urgent need to develop strategies for prevention and infection control. Limiting the use of certain antimicrobials may be an effective strategy for curbing the spread of this pathogen.

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