Nosocomial Outbreak of Extensively Drug-Resistant (Polymyxin B and Carbapenem) Klebsiella pneumoniae in a Collapsed University Hospital Due to COVID-19 Pandemic

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Abstract: We correlated clinical, epidemiological, microbiological, and genomic data of an outbreak with polymyxin B (PB)- and carbapenem-resistant Klebsiella pneumoniae during the COVID-19 pandemic. Twenty-six PB- and carbapenem-resistant K. pneumoniae were isolated from patients in the COVID-19 ICU (Intensive Care Unit), non-COVID-19 ICU (Intensive Care Unit), clinical, or surgical ward. Bacterial identification, drug susceptibility tests, and DNA sequencing were performed, followed by in silico resistance genes identification. All isolates showed extensively drug-resistant (XDR) phenotypes. Four different sequence types (ST) were detected: ST16, ST11, ST258, and ST437. Nineteen isolates were responsible for an outbreak in the ICU in September 2020. They belong to ST258 and harbored the 42Kb IncX3 plasmid (pKP98M3N42) with the same genomic pattern of two K. pneumoniae identified in 2018. Twenty-four isolates carried bla_KPC-2 gene. No plasmid-mediated colistin (mcr) resistance genes were found. Eight isolates presented mgrB gene mutation. The clonal isolates responsible for the outbreak came from patients submitted to pronation, with high mortality rates in one month. XDR-K. pneumoniae detected during the outbreak presented chromosomal resistance to PB and plasmid-acquired carbapenem resistance due to KPC production in most isolates and 42Kb IncX3(pKP98M3N42) plasmid carrying bla_KPC-2 was associated with ST258 isolates. The outbreak followed the collapse of the local healthcare system with high mortality rates.

Keywords: clinical evaluation; polymyxin resistance; extensively drug-resistance; multidrug-resistance; molecular epidemiology; KPC

1. Introduction

The first cases of COVID-19 in Brazil were reported on 25 February 2020. One year later, more than half a million deaths were reported due to COVID-19, most of them after a long stay in intensive care units (ICU). Severe cases were described to be frequently associated with comorbidities, age above 50, exacerbated inflammatory response, and invasive procedures (mechanical ventilation, central intravenous access), which facilitated...
healthcare-associated infections (HAI) [1]. Hospitalized patients with COVID-19 and bacterial secondary infections have commonly been detected, resulting in high rates of antibiotic use (up to 94 to 100%) among patients with confirmed cases of COVID-19 [2]. Several studies showed an increase in antimicrobial resistance during the COVID-19 pandemic, mainly related to Gram-negative bacilli, driven by abusive antimicrobial use, overload of health professionals, and prolonged time with invasive therapy.

Antimicrobial resistance during the COVID-19 pandemic is highly associated with the presence of *Acinetobacter baumannii* and *Klebsiella pneumoniae*, mainly carbapenemase-producing (e.g., KPC) isolates [3,4]. A review evaluating the impact of the ongoing pandemic on antimicrobial resistance showed that the vast majority of the reports came from ICU. Reports of clusters of infections due to the same resistant pathogen raised the concern of a decline in adherence to infection control and prevention measures. Another aspect is the importance of environmental contamination [4].

A previous study in our hospital, from 2018 to 2020, showed an increase in the rate of *A. baumannii* and *K. pneumoniae* resistant to carbapenem per 1000 patients/day after the beginning of the COVID-19 pandemic. There was a 10-fold increase (from 5% to 50%) in the number of polymyxin B (PB)-resistant *K. pneumoniae* isolation comparing 2018–2019 (before COVID-19) to the same period after the pandemic onset [5].

This study aimed to characterize and correlate clinical, epidemiological, microbiological, and genomic data from an outbreak of PB- and carbapenem-resistant *K. pneumoniae* isolated from patients in a tertiary hospital during the collapse of the healthcare system in 2020 due to the COVID-19 pandemic.

2. Materials and Methods

2.1. Study Design

We included 26 PB- and carbapenem-resistant *K. pneumoniae* isolated from patients in a hospital with 551 beds just after healthcare collapse due to COVID-19. Patients were admitted into one of the four medical units: COVID-19 ICU, non-COVID-19 ICU, clinical, or surgical ward. The genomic profile of 2020 *K. pneumoniae* detected during COVID-19 pandemic were compared with isolates sequenced in 2018 in the same hospital, which is the reference for 3.5 million people in Southern Brazil (Ribeirão Preto region of São Paulo State).

This study was approved by the Ethics Committee of the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto-Universidade de São Paulo (CAAE: 51569821.9.0000.5440).

2.2. Clinical Data and Context

Clinical data were collected from 1 September to 30 November 2020. COVID-19's first peak occurred from June to August 2020 in Brazil. The following information was collected: gender, age, underlying disease, SARS-CoV-2 infection, hospitalization time, HAI, hospital unit where *K. pneumoniae* were isolated, previous use of polymyxin B (PB), and other antibiotics. The clinical samples were diagnostic of hospital infection, and the samples included in the study were: blood, tracheal secretion, urine, peritoneal fluid, and surgical wound.

2.3. Identification and Antimicrobial Susceptibility Testing of Isolates

The identification of bacterial isolates and the antimicrobial susceptibility testing were performed by Vitek®2 (bioMérieux, Marcy l’Etoile, France). Ceftazidime-avibactam (CZA) susceptibility using ETEST® (bioMérieux, Marcy l’Etoile, France) or by standardized disk diffusion method. PB minimum inhibitory concentration (MIC) was first determined using the commercial test based on a panel with lyophilized PB, Policimbac® (Probac, São Paulo, Brazil) [6].

PB MIC was confirmed using the reference method of broth microdilution assay [7]. Water solution of PB (United States Pharmacopeia, Rockville, United States of America), Cation-Adjusted Mueller Hinton II Broth (CAMHB) (BBL™, Becton Dickinson, New Jersey, United States of America), and 96-well microplates polystyrene, round bottom, non-treated,
were used for assays. Two-fold serial dilution (256–0.5 µg/mL) of PB was evaluated and MIC values were determined as the lowest concentrations of PB that inhibit visible bacterial growth in CAMHB. *Escherichia coli* ATCC 25,922 and *E. coli* NCTC 13,846 (*mcr-1* positive) were used as quality control. The strains were considered PB-resistant when MIC was higher than 2 mg/L, according to EUCAST [7].

2.4. DNA Extraction and Genome Sequencing

Total genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. The DNA concentrations were measured fluorometrically (Qubit® 3.0, kit Qubit® dsDNA Broad Range Assay kit, Life Technologies, Carlsbad, CA, USA). Purified DNA was prepared for sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). Libraries were assessed for quality using the 2100 Bioanalyzer (Agilent Genomics, Santa Clara, CA, USA). DNA sequencing was performed at Novogene (Sacramento, CA, USA) on a Novaseq6000 platform. Adapters were trimmed using Trimmomatic v0.36. Samples were evaluated for possible contamination using Bowtie2 v2.2.3. Overlapped reads were merged using Flash version 1.2.11. Merged and unmerged reads were assembled using Spades v3.12.0. Genome quality (completeness and contamination) was evaluated using CheckM v1.0.7 and QUAST. Genome annotations were performed using Prokka v1.11 with default parameters. The amino acid sequences of all genes identified, using Prokka, were aligned with the National Database of Antibiotic Resistant Organisms (NDARO) database obtained from NCBI (March 2021).

The alignment was performed using Diamond v0.8.24. Alignments with ≥60 similarity score were selected for further analysis. Final quality of genome assembly was determined using MIGA web service. Only samples with High Quality (>80%), High Level of Completeness (>95%), and Low Contamination (<3%) were selected for further analysis. All genomes are available at the NCBI under the BioProject number PRJNA755436.

2.5. In Silico Identification of Carbapenem and Polymyxin B Resistance Genes and Characterization of Bacterial Genomic Relationship

The identification of acquired carbapenemase resistance genes was performed using the ABRICATE pipeline by searching annotated genes using reference databases (ARG-ANNOT, NCBI AMRFinderPlus, CARD, and ResFinder). Chromosomal and plasmid localization of resistance genes was evaluated using ARG information from ARG-ANNOT [8]. Identification of plasmid-carrying carbapenemase genes was performed using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/ (accessed on 19 March 2021).

Chromosomal genes related to PB resistance were also investigated [3,9–11]. Genomes of PB-resistant *K. pneumoniae* clinical isolates studied were compared to the genome of PB-susceptible and KPC-producing *K. pneumoniae* ATCC BAA-1705 standard strain (https://www.patricbrc.org/view/Genome/1276652.3 (accessed on 30 April 2021). Mutations were manually evaluated and annotated for each clinical isolate studied.

Phylogenomic analyses were performed using Parsnp and Ginger. Phylogenomic trees were produced by iTOL. Data manipulation was completely performed in Python (3.7.4), all scripts can be found in the git repository (https://github.com/tamascogustavo/project_MDR_KRP (accessed on 31 August 2021). Sequence type (ST) determination was carried out in the *Klebsiella* Multi-Locus Sequence Typing (MLST) database, from Institute Pasteur, based on whole genome sequence [12].

3. Results

3.1. *K. pneumoniae* Clinical Isolates Features

Based on the SNP’s analysis, four groups were detected, and they were related to the sequence types ST11, ST16, ST258, and ST437 with a predominance of the clonal group.
Among the 26 *K. pneumoniae* initially detected as PB-resistant, 20 were confirmed by broth microdilution assay. No plasmid-mediated colistin (*mcr*) resistance genes were found. Eight isolates (30.8%) presented mutation in *mgrB* gene, *pmrA* gene mutation was found in most isolates (96.1%) except L14, and none presented mutations in *phoP*, *phoQ*, or *pagP* genes (Table 1). Mutations in those genes result in the addition of cationic groups in the lipopolysaccharide (LPS), leading to the repulsion of polymyxin molecule, which results in polymyxin resistance [14].

Table 1. *K. pneumoniae* isolates susceptibility pattern and mutated genes related to PB resistance.

| Period       | Clinical Setting | Date     | ID  | ST | PB MIC (µg/mL) | Mutated Genes Related to PB Resistance |
|--------------|-----------------|----------|-----|----|----------------|---------------------------------------|
|              |                 |          |     |    | Policimac      | Broth Microdilution                     |
| October 2020 | COVID ICU       | 7        | L19 | 16 | ≥64            | 32                                    |
|              | Clinical Ward   | 5        | L20 | 258| 32             | 2                                     |
|              | Surgical Ward   | 8        | L27 | 258| 4              | 2                                     |
|              |                 | 24       | L24 | 258| 4              | 2                                     |
|              |                 | 3        | L26 | 11 | ≥64            | 128                                   |
|              | November 2020   | COVID ICU| 7   | L19| 16             | 32                                    |
|              | Clinical Ward   | 5        | L20 | 258| 32             | 8                                     |

ICU: intensive care units; ID: sample identification; ST: sequence type; PB: polymyxin B; MIC: minimal inhibitory concentration.
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3.1. K. pneumoniae Clinical Isolates Features

Based on the SNP’s analysis, four groups were detected, and they were related to the sequence types ST11, ST16, ST258, and ST437 with a predominance of the clonal group (CG) 258 (Figure 1). All the isolates were extensively drug-resistant (XDR) [13], as shown in Table S1.

Among the 26 K. pneumoniae initially detected as PB-resistant, 20 were confirmed by broth microdilution assay. No plasmid-mediated colistin (mcr) resistance genes were found. Eight isolates (30.8%) presented mutation in mgrB gene, pmrA gene mutation was found in most isolates (96.1%) except L14, and none presented mutations in phoP, phoQ, or pagP genes (Table 1). Mutations in those genes result in the addition of cationic groups in the lipopolysaccharide (LPS), leading to the repulsion of polymyxin molecule, which results in polymyxin resistance [14].

Figure 1. Phylogenomic tree of 26 K. pneumoniae isolated in 2020 (black) during the COVID-19 pandemic, with five isolates sequenced in 2018 in the same hospital (light blue), based on SNP detection. Isolates from the “orange group” belong to ST258 and isolates from the “green and blue groups” belong to the ST11. Bacteria holding plasmid pkP98M3N42 with coverage scores over 90% and similarity scores over 99% with the reference [15] are indicated with a green solid circle.

K. pneumoniae L13 and L04 isolates belong to the same ST11 clone (Table 1 and Figure 1). They presented resistance to ertapenem (ERT), intermediate resistance (susceptible with increased exposure) to meropenem (MER), and susceptibility to imipenem (IPM). Only these two isolates did not harbor the blaKPC-2 gene (or another carbapenemase gene). Additionally, L13 and L04 isolates showed different susceptibility to PB (respectively, MIC = 32 µg/mL, and MIC = ≤ 0.5 µg/mL) as well as different mutation in genes related to PB susceptibility (respectively, mgrB, pmrA, pmrD, and pmrA, pmrD, ramA).

The 42 Kb IncX3 plasmid (pKP98M3N42) was identified on 19/26 (73%) isolates from ST258 and in L28 isolate belonging to ST11, harboring blaKPC-2 resistance determinant (coding for K. pneumoniae carbapenemase [KPC], a beta-lactamase that may confer resistance to all beta-lactam antibiotics, highlighted carbapenems) (Figure 1). In addition, transposases, recombinases, and type IV secretion system genes were found in this plasmid [15].

Other different resistance determinants were found, including broad spectrum and extended spectrum beta-lactamase (ESBL) genes (blaSHV and blaCTX-M), plasmid-mediated quinolone resistance gene (qnr), fosfomycin resistance gene (fos), aminoglycoside modifying enzymes (AMEs), and 16S rRNA methylases enzymes resistance genes (rmt, aac, aad, ant), and tetracyclines resistance gene (tet) (Table S2 and Figure S1).
3.2. Clinical Features and Patients’ Outcomes

The number of COVID-19 patients and ICU beds in the hospital increased exponentially after March 2020. The number of patients in the COVID-19 ICU increased from zero (March) to 128 (August 2020) (Figure 2). Concurrently, the number of COVID-19 ICU beds was increased from the initial 21 to 89 in four ICUs in the same period. The number of \textit{K. pneumoniae} resistant to PB isolated in the hospital raised from 100 in 2019, to 350 (2020) and 450 till August 2021 (Figure S2).

![Figure 2](https://ourworldindata.org/coronavirus/country/brazil)

The incidence density for PB-resistant \textit{K. pneumoniae} also demonstrates an increase in the pandemic period related to the pre-pandemic period (18.5 positive samples for PB-resistant \textit{K. pneumoniae}/1000 patients/day versus 1.5 positive samples for PB-resistant \textit{K. pneumoniae}/1000 patients/day). The sites of infection are described in Table 2. Eight (30.8%) \textit{K. pneumoniae} in the study were considered colonization. In the other 18 patients (69.2%) \textit{K. pneumoniae} was the cause of HAI.

Most (80%) patients who died had at least one comorbidity with cardiovascular diseases being predominant. Among 26 selected patients, eight (30.7%) died: five in September, two in October, and two in November. All the patients received antibiotics treatment that included one or more of the following: azithromycin (previous use of antimicrobial during hospitalization before isolation of the extensively drug-resistant \textit{Klebsiella pneumoniae}.) ceftriaxone, cefepime, piperacillin-tazobactam, aminoglycosides, and carbapenem.

Two-thirds (16) of PB- and carbapenem-resistant \textit{K. pneumoniae} isolates were detected in September 2020 and ten (38%) in October and November 2020 (Figure 3). The COVID-19 first wave peak in Southern Brazil occurred from June to August 2020, when the healthcare system collapsed for the first time (Figure 2).
In September 2020, 17 PB- and carbapenem-resistant *K. pneumoniae* were isolated in the hospital, and most of them, 11 (64.7%), were recovered from patients in the COVID-19 ICU. During this month, 15 out of 17 (88.2%) isolates were defined as ST258, with nine patients in the COVID-19 ICU, two in non-COVID ICU, two in the clinical, and two in the surgical ward. Six out of 11 (54.5%) COVID-19 patients in the ICU died, and five (80%) were from ST258. Fifty percent of the ST258 isolates were from patients who required pronation during COVID-19 management in the ICU. Six out of 10 (60%) patients with *K. pneumoniae* ST258 infections died in the COVID-19 ICU during September (Figure 3 and Table 2).

From October to November 2020, a homogeneous distribution of *K. pneumoniae* infection cases could be observed among clinical settings. ST258 was the predominant lineage with 50% of the cases (Table 1). Three deaths were observed during this period, two in the non-COVID ICU and one in the clinical ward (Figure 3).

In this period, there was an increase in healthcare-related infection rates, the main ones being ventilator-associated pneumonia and primary bloodstream infection (Table 2). The HAI was computed among patients with KPC infection. Figure 2B lists the COVID-19 deaths in the year 2020 in Brazil.

### Table 2. Demographic characteristics of 26 patients with positive culture for polymyxin B- and carbapenem-resistant *K. pneumoniae* in the period of September to November 2020.

| Demographics Characteristics | Results |
|-----------------------------|---------|
| Mean age (Min-Max)          | 57.8 (21–85) |
| Gender (n = 26)             | n (%) |
| Male                        | 15 (57.6) |
| Female                      | 11 (42.3) |
| Admission Unit (n = 26)     | n (%) |
| COVID ICU                   | 13 (50.2) |
| Non-COVID ICU               | 4 (15.3) |
| Clinical Ward               | 5 (19.2) |
| Surgical Ward               | 4 (15.3) |
| Hospitalization days median (max-min) | 49 (110–8) |
| Comorbidities (n = 26)      | n (%) |
| Systemic Arterial Hypertension (SAH) | 10 (40) |
| Diabetes Mellitus (DM)      | 4 (16) |
| Obesity                     | 2 (8) |
| Other diseases              | 9 (36) |
| Healthcare-associated infections (n = 18) | n (%) |
| Bloodstream infection       | 6 (33.3) |
| Ventilator-Associated Pneumonia | 5 (27.8) |
| Urinary Tract Infection     | 3 (16.6) |
| Surgical Site Infection     | 2 (11.1) |
| Peritonitis                 | 1 (5.6) |
| Tracheitis                  | 1 (5.6) |
| COVID-19 infection          | n (%) |
| Positive                    | 19 (73) |
| Negative                    | 6 (23) |
| Inconclusive                | 1 (4) |
| Passage by the intensive care unit n (%) | 21 (80.7) |
| Pronation n (%)             | 7 (26.9) |
| Previous use of polymyxin n (%) | 13 (50) |

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In this period, there was an increase in healthcare-related infection rates, the main ones being ventilator-associated pneumonia and primary bloodstream infection (Table 2). The HAI was computed among patients with KPC infection. Figure 2B lists the COVID-19 deaths in the year 2020 in Brazil.
Figure 3. Schematic representation of the distribution of the 26 K. pneumoniae isolates recovered from hospitalized patients from September to November 2020 in the COVID-19 ICU, Non-COVID-19 ICU, Clinical Ward, and Surgical Ward. Legends represent the color code for the sequence type (ST), presence of 42 Kb IncX3 plasmid (pKP98M3N42) harboring \( \text{bla}_\text{KPC-2} \) (green solid circle), pronation practices in hospitalized patients (orange line), and death of the patient (red cross).

4. Discussion

Our investigation of the nosocomial outbreak of extensively drug-resistant K. pneumoniae during the COVID-19 pandemic revealed a concerning scenario. The rapid spread of SARS-CoV-2 and the increased number of severe cases resulted in the system’s collapse. An overcrowded ICU, lack of specialized healthcare professionals, and other healthcare resources during the peak of COVID-19 aggravated the situation. Tiri et al. (2020) observed an increase in the number of Carbapenem-resistant Enterobacteriaceae (CRE) at the University Hospital of Terni (Italy). Analyzing patients colonized with CRE on a quarterly basis, the authors observed the incidence of CRE from 6.7% in 2019 to 50% in March/April 2020, at the peak of the COVID-19 pandemic. High workload of professionals, prone position, lack of professional experience in the ICU setting, and overuse of antimicrobials may have accounted for this increase during the pandemic [16]. The same was observed in our study in the COVID-19 ICUs during September 2020.

Adebsi et al. (2021) had already evidenced that secondary bacterial infections play crucial roles in mortality and morbidity associated with COVID-19 [17]. The challenge to differentiate bacterial- from COVID-19 pneumonia and the absence of antiviral therapy with confirmed efficacy and the urgent need to start some therapy increase the use of antibiotics as part of the strategy to manage COVID-19 critical patients [18]. A study in Peru reported that 68.9% of patients with COVID-19 received antibiotics (mainly azithromycin and ceftriaxone), and a self-medication rate of 33%, before hospital admission [19].

XDR clinical isolates, especially co-resistant to carbapenems, aminoglycosides, polymyxins (e.g., PB), and tigecycline (CAPT-resistant) have been increasingly reported worldwide and represent a real possibility of dissemination of pandrug-resistant Gram-negative bacilli.
KPC-2-producing *K. pneumoniae* CG258 (ST258 and ST11 international high-risk clones, as well as ST437) have been circulating all over the world and in our hospital, at least, since 2007 initially represented by a clonal outbreak of ST258 [20–22].

PB-resistant and KPC-2-producing *K. pneumoniae* ST11 emerged and was firstly detected in 2012. These isolates were characterized by a clonal outbreak that also contained the same ST11 clone susceptible to PB, showing an initial diversification and selection of PB-resistant ST11 clones in our hospital.

The outbreak isolates were compared to previous *K. pneumoniae* identified in the same hospital two years prior and two (98M3 and 125M3) belong to ST258 which harbors the 42 Kb IncX3 plasmid (pKP98M3N42), with the exact pattern observed in 19 *K. pneumoniae* that was responsible for the outbreak in the COVID-19 ICU in September 2020 [10]. *K. pneumoniae* isolate L28 also harbors 42 Kb IncX3 plasmid but belongs to a different ST (ST11; blue group); however, it is part of the same clonal group (CG258) shared by the 19 *K. pneumoniae* isolates gathered in the orange group (Figure 1).

The CG258 clonal group includes ST258, ST11, ST340, ST437, and ST512 [23], and it is related to the acceleration of spread in nosocomial outbreaks when associated with the resistance gene, which is comprised in the pKP98M3N42 plasmid [24].

This epidemiological scenario is similar worldwide and the association of the gene *bla*KPC-2 and CG258 isolates have boosted the outbreaks as well as endemicity of these bacteria [25].

We observed five infections due to ST11 isolates resulting in three deaths. Jin et al. (2021) analyzed 11 isolates of carbapenem-resistant *K. pneumoniae* with a *bla*KPC-2 located in a same size plasmid. This is a potential threat to anti-infective treatment and is capable of rapid and diverse evolution of resistance during treatment with tigecycline and polymyxin [26]. Mutations associated with the mgrB gene, detected in eight isolates in this study (Table 1), are the most common cause of polymyxin resistance in *K. pneumoniae* [27,28].

Although mutations in the pmrA gene were detected in most of the 26 isolates, it was described that only a few mutations on this gene are responsible for PB resistance [28]. This might explain why some isolates with mutations in pmrA were susceptible to PB (Table 1).

The 42 Kb IncX3 plasmid (pKP98M3N42), harboring *bla*KPC-2, has also been reported in bacterial strains in Brazil since 2009 [15] and plays an important role in horizontal gene transfer conferring resistance to carbapenems [23] and polymyxins [10].

Interesting, *K. pneumoniae* L13 and L04 isolates belong to the same ST11 clone and showed different PB susceptibility, mutations in genes related to PB susceptibility, and different dates and places of isolation in the hospital. Thereby, these characteristics and scenarios suggest intra-clonal diversification and evolution of the L04 isolate (mutation in mgrB gene and PB MIC = 32 µg/mL) (Table 1).

From July to September 2020, COVID-19 ICU had 100% occupancy, despite the number of beds increasing more than four times. These new ICU places were created due to the high number of COVID-19 critical cases. In September 2020, a likely association can be observed between high mortality and infections due to PB- and carbapenem-resistant *K. pneumoniae* ST258, carrying 42Kb-IncX3-*bla*KPC-2 in the COVID ICU. In the following two months, the distribution of cases among clinical wards changed and the number of isolates decreased significantly; however, ST258 was still predominant.

As several isolates are identical by genomic sequencing, we can assume (hypothesis) that this isolate has increased in frequency in the ICU COVID because of the difficulties of treatment and control of the isolate. As there are patients who are discharged from the ICU to wards, this isolate may have been taken to these other places, or the other possibility is that due to the overload of work faced by the teams, many doctors and nurses were required to cover more than one unit in a rotation scheme, moving them between different units during the worst period of the pandemic (Figure 3).

This study has some limitations as we analyzed only positive clinical samples with *K. pneumoniae* resistant to carbapenem and PB, and we did not measure prior use of antimicrobials other than meropenem and PB. It was not possible to analyze the adherence
to infection control preventive measures (e.g., hand hygiene rate) during the study period. Finally, this is a single setting reality and may not be generalizable to other contexts.

In summary, COVID-19, coupled with an XDR-\textit{K. pneumoniae} outbreak with high mortality rates, collapsed the local healthcare system not only in the COVID ICU but in different clinical settings in the hospital. In addition, the high labor demand may have favored the cross-transmission of this XDR \textit{K. pneumoniae}, increasing antimicrobial consumption during the COVID-19 pandemic.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11060814/s1, Figure S1: Heatmap showing the presence of ARGs identified by ARG-ANNOT indicating the number of each resistance gene per genome. Data were clustered using hierarchical mapping with Euclidian distance. Blue to red scale indicates number of ARG for each strain in each category, as indicated in the legend. The nucleotide sequences in ARG-ANNOT from different antibiotics are abbreviated as follows: AGly, aminoglycosides resistance genes; Bla, beta-lactamases; Fcyr, fosfomycin; Flq, fluoroquinolones; Gly, glycopeptides; MLS, macrolide-lincosamide-streptogramin; Phe, phenicol; Rif, rifampin; Sul, sulfonamides; Tet, tetracyclines; and Tmt, trimethoprim. Bacteria holding plasmid pKp98M3N42 with coverage scores over 90% and similarity scores over 99% with the reference are indicated with a green solid circle; Figure S2: Number of \textit{Klebsiella pneumoniae} isolates resistant to polymyxin and carbapenem in the Ribeirão Preto Clinics Hospital in 2019, 2020 and 2021 (Jan-Aug). Many patients had more than one isolate identified with the same drug-susceptibility profile. In 2021 there were 3.5 times more drug-resistant isolates than in 2019. During 2020 and 2021 the increment was quite related to the healthcare system collapse due to COVID-19 pandemic; Table S1: Antibiotic resistance profile of 26 PB- and carbapenem-resistant \textit{K. pneumoniae} isolated from clinical patients during COVID-19 outbreak; Table S2: Acquired genes conferring resistance to beta-lactams, tetracyclines, aminoglycosides, fosfomycin, and fluoroquinolones found in the isolates genome.

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**References**

1. Ranzani, O.T.; Bastos, L.S.L.; Gelli, J.G.M.; Marchesi, J.F.; Biaió, F.; Hamacher, S.; Bozza, F.A. Characterisation of the First 250,000 Hospital Admissions for COVID-19 in Brazil: A Retrospective Analysis of Nationwide Data. \textit{Lancet Respir. Med.} 2021, 9, 407–418. [CrossRef]

2. Murray, A.K. The Novel Coronavirus COVID-19 Outbreak: Global Implications for Antimicrobial Resistance. \textit{Front. Microbiol.} 2020, 11, 1020. [CrossRef] [PubMed]

3. Cantón, R.; Gijón, D.; Ruiz-Garbajosa, P. Antimicrobial Resistance in ICUs: An Update in the Light of the COVID-19 Pandemic. \textit{Curr. Opin. Crit. Care} 2020, 26, 433–441. [CrossRef]

4. Segala, F.V.; Bavaro, D.F.; di Gennaro, F.; Salvati, F.; Marotta, C.; Saracino, A.; Murri, R.; Fantoni, M. Impact of SARS-CoV-2 Epidemic on Antimicrobial Resistance: A Literature Review. \textit{Viruses} 2021, 13, 2110. [CrossRef] [PubMed]
5. Gaspar, G.G.; Ferreira, L.R.; Feliciano, C.S.; Campos Júnior, C.P.; Molina, F.M.R.; Vendruscolo, A.C.S.; Bradan, G.M.A.; Lopes, N.A.P.; Martinez, R.; Bollela, V.R. Pre- and Post-COVID-19 Evaluation of Antimicrobial Susceptibility for Healthcare-Associated Infections in the Intensive Care Unit of a Tertiary Hospital. Rev. Soc. Bras. Med. Trop. 2021, 54. [CrossRef] [PubMed]

6. Dalmolin, T.V.; Carneiro, M.D.S.; de Castro, L.P.; Volpato, E.C.Z.; Wink, P.L.; de Lima-Morales, D.; Barth, A.L. Evaluation of the Susceptibility Test of Polymyxin B Using the Commercial Test Polimixbac®. Braz. J. Microbiol. 2020, 51, 1135–1137. [CrossRef]

7. EUCAST. Antimicrobial Susceptibility Testing. Available online: https://www.eucast.org/ast_of_bacteria/ (accessed on 9 April 2022).

8. Gupta, S.K.; Padmanabhan, B.R.; Diene, S.M.; Lopez-Rojas, R.; Kempf, M.; Landraud, L.; Rolain, J.-M. ARG-ANNOT, a New Bioinformatic Tool to Discover Antibiotic Resistance Genes in Bacterial Genomes. Antimicrob. Agents Chemother. 2014, 58, 212–220. [CrossRef]

9. Wright, M.S.; Suzuki, Y.; Jones, M.B.; Marshall, S.H.; Rudin, S.D.; van Duin, D.; Kaye, K.; Jacobs, M.R.; Bonomo, R.A.; Adams, M.D. Genomic and Transcriptomic Analyses of Colistin-Resistant Clinical Isolates of Klebsiella Pneumoniae Reveal Multiple Pathways of Resistance. Antimicrob. Agents Chemother. 2015, 59, 536–543. [CrossRef]

10. Fernandes, M.R.; McCulloch, J.A.; Vianello, M.A.; Moura, Q.; Perez-Chaparro, P.J.; Esposito, E.; Sartori, L.; Dropa, M.; Mattei, M.H.; Lira, D.P.A.; et al. First Report of the Globally Disseminated IncX4 Plasmid Carrying the Mcr-1 Gene in a Colistin-Resistant Escherichia Coil Sequence Type 101 Isolate from a Human Infection in Brazil. Antimicrob. Agents Chemother. 2016, 60, 6415–6417. [CrossRef]

11. Campos, P.A.D.; Fuga, B.; Ferreira, M.L.; Brigido, R.T.E.S.; Lincopan, N.; Gontijo-Filho, P.P.; Ribas, R.M. Genetic Alterations Associated with Polymyxin B Resistance in Nosocomial KPC-2-Producing Klebsiella Pneumoniae from Brazil. Microb. Drug Resist. 2021, 27, 1677–1684. [CrossRef]

12. Pasteur Institute. Klebsiella Sequence Typing. Available online: https://bigsdb.pasteur.fr/klebsiella/ (accessed on 9 April 2022).

13. Magiorakos, A.P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Harbarth, S.; Hindler, J.F.; Kahlmeter, G.; Hunt, P.; Just, J.; et al. Multidrug-Resistant, Extensively Drug-Resistant and Pandrug-Resistant Bacteria: An International Expert Proposal for Interim Standard Definitions for Acquired Resistance. Clin. Micro-Biol. Infect. 2012, 18, 268–281. [CrossRef]

14. Poirel, L.; Gayol, A.; Nordmann, P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. Clin. Microbiol. Rev. 2017, 30, 557–596. [CrossRef] [PubMed]

15. Borelli, T.C.; Lovate, G.L.; Scaranello, A.F.T.; Ribeiro, L.F.; Zaramela, L.; Pereira-dos-Santos, F.M.; Silva-Rocha, R.; Guazzaroni, M.E. Combining Functional Genomics and Whole-Genome Sequencing to Detect Antibiotic Resistance Genes in Bacterial Strains Co-Occurring Simultaneously in a Brazilian Hospital. Antibiotics 2021, 10, 419. [CrossRef] [PubMed]

16. Tiri, B.; Sensi, E.; Marsiliani, V.; Cantarini, M.; Priante, G.; Verneli, C.; Martella, L.A.; Costantini, M.; Mariottini, A.; Andreani, P.; et al. Antimicrobial Stewardship Program, COVID-19, and Infection Control: Spread of Car-bapenem-Resistant Klebsiella Pneumoniae Colonization in ICU COVID-19 Patients. What Did Not Work? J. Clin. Med. 2020, 9, 2744. [CrossRef] [PubMed]

17. Adebesi, Y.A.; Alaran, A.J.; Okereke, M.; Oke, G.I.; Oladeji, O.C.; Oladunjoye, I.; Olarewaju, A.Y.; Ukor, N.A.; Lucero-Prisno, D.E. COVID-19 and Antimicrobial Resistance: A Review. Infectious Diseases. Res. Treat. 2021, 14, 117863372110338. [CrossRef] [PubMed]

18. Ginsburg, A.S.; Klugman, K.P. COVID-19 Pneumonia and the Appropriate Use of Antibiotics. Lancet Glob. Health 2020, 8, e1453–e1454. [CrossRef]

19. Zavala-Flores, E.; Salcedo-Matienzo, J. Medicación Prehospitalaria En Pacientes Hospitalizados Por COVID-19 En Un Hospital Público de Lima-Perú. Acta Med. Peru. 2020, 37, 393–395. [CrossRef]

20. Andrade, L.N.; Vitali, L.; Gaspar, G.G.; Bellissimo-Rodrigues, F.; Martinez, R.; Darini, A.L.C. Expansion and Evolution of a Virulent, Extensively Drug-Resistant (Polymyxin B-Resistant), QnrS1-, CTX-M-2-, and KPC-2-Producing Klebsiella Pneumoniae ST11 International High-Risk Clone. J. Clin. Microbiol. 2014, 52, 2530–2535. [CrossRef]

21. Andrade, L.N.; Curiao, T.; Ferreira, J.C.; Longo, J.M.; Climaco, E.C.; Martinez, R.; Bellissimo-Rodrigues, F.; Basile-Filho, A.; Evaristo, M.A.; del Peleos, P.F.; et al. Dissemination of Bla KPC-2 by the Spread of Klebsiella Pneumoniae Clonal Complex 258 Clones (ST258, ST11, ST437) and Plasmids (IncFII, IncN, IncL/M) among Enterobacteriaceae Species in Brazil. Antimicrob. Agents Chemother. 2011, 55, 3579–3583. [CrossRef] [PubMed]

22. Gaspar, G.G.; Bellissimo-Rodrigues, F.; de Andrade, L.N.; Darini, A.L.; Martinez, R. Induction and Nosocomial Dissemination of Carbapenem and Polymyxin-Resistant Klebsiella Pneumoniae. Rev. Soc. Bras. Med. Trop. 2015, 48, 483–487. [CrossRef]

23. Cerdeira, L.T.; Cunha, M.P.V.; Francisco, G.R.; Bueno, M.F.C.; Araujo, B.F.; Ribas, R.M.; Gontijo-Filho, P.P.; Knobl, T.; de Oliveira Garcia, D.; Lincopan, N. IncX3 Plasmid Harboring a Non-Te 4401 Genetic Element (NTE KPC) in a Hospital-Associated Clone of KPC-2-Producing Klebsiella Pneumoniae ST340/CG258. Diagn. Microbiol. Infect Tious Dis. 2017, 89, 164–167. [CrossRef] [PubMed]

24. Tofteland, S.; Naseer, U.; Lislelvd, J.H.; Sundsfjord, A.; Samuelsen, Ø. A Long-Term Low-Frequency Hospital Outbreak of KPC-Producing Klebsiella Pneumoniae Involving Intergenus Plasmid Diffusion and a Persisting Envi-ronmental Reservoir. PLoS ONE 2013, 8, e59015. [CrossRef] [PubMed]

25. Andrey, D.O.; Pereira Dantas, P.; Martins, W.B.; De Carvalho, F.M.; Almeida, L.G.P.; Sands, K.; Portal, E.; Sauser, J.; Cayó, R.; Nicolás, M.F.; et al. An Emerging Clone, Klebsiellapneumoniae Carbapenemase-2-Producing K. Pneumoniae Sequence Type 16, Associated With High Mortality Rates in a CC258-Endemic Setting. Clin. Infect. Dis. 2020, 71, e141–e150. [CrossRef] [PubMed]
26. Jin, X.; Chen, Q.; Shen, F.; Jiang, Y.; Wu, X.; Hua, X.; Fu, Y.; Yu, Y. Resistance Evolution of Hypervirulent Car-bapenem-Resistant Klebsiella Pneumoniae ST11 during Treatment with Tigecycline and Polymyxin. Emerg. Microbes Infect. 2021, 10, 1129–1136. [CrossRef] [PubMed]

27. Cannatelli, A.; D’Andrea, M.M.; Giani, T.; di Pilato, V.; Arena, F.; Ambretti, S.; Gaibani, P.; Rossolini, G.M. In Vivo Emergence of Colistin Resistance in Klebsiella Pneumoniae Producing KPC-Type Carbapenemases Mediated by in-sertional Inactivation of the PhoQ/PhoP MgrB Regulator. Antimicrob. Agents Chemother. 2013, 57, 5521–5526. [CrossRef] [PubMed]

28. Olaitan, A.O.; Morand, S.; Rolain, J.-M. Mechanisms of Polymyxin Resistance: Acquired and Intrinsic Resistance in Bacteria. Front. Microbiol. 2014, 5, 643. [CrossRef] [PubMed]