Occurrence of Klebsiella pneumoniae ST244 and ST11 extensively drug-resistant producing KPC, NDM, OXA-370 in wastewater, Brazil

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Abstract

Aims: To investigate the occurrence of carbapenem-resistant Klebsiella pneumoniae (CRKP) and their clonal relationships from hospital and municipal wastewater treatment plants (WWTPs). Methods and Results: Eighteen K. pneumoniae strains recovered from three WWTPs were identified by MALDI-TOF. The antimicrobial susceptibility were evaluated by disk-diffusion and the carbapenemases production by Carbapenembac®. The carbapenemases genes were investigated by real-time PCR and the clonal relationship through multi-locus sequence typing (MLST). Thirty nine percent (7/18) of isolates were classified as multidrug-resistant (MDR), 61.1% (11/18) extensively drug-resistant (XDR) and 83.3% (15/18) showed carbapenemase activity. Three carbapenemase-encoding genes were found, \textit{blaKPC} (55%), \textit{blaNDM} (27.8%) and \textit{blaOXA-370} (11.1%) as well five sequencing types ST11, ST37, ST147, ST244 and ST281. ST11 and ST244; sharing four alleles were grouped into clonal complex 11 (CC11). Conclusions: Our results show the importance of monitoring antimicrobial resistance in WWTPs effluents to minimize the risk of spreading bacterial load and ARGs in aquatic ecosystems, using advanced treatment technologies to reduce these emerging pollutants at WWTPs. Significance and Impact of Study: To our knowledge, this is the first report of KPC, NDM, OXA-370- producing ST 244 in sewage. Furthermore, the occurrence and persistence of CRKP ST11 carrying different resistance genetic profiles in treated effluents may generating negative impacts on public and environmental health.

Keywords: Klebsiella pneumoniae, Wastewater, Antibiotic resistance genes, carbapenemase, multilocus sequencing typing.
1. Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains are constantly associated with nosocomial infections (DAVID et al., 2019; LI et al., 2022). The spread of high-risk clones is considered a worldwide potential threat (ARCARI; CARATTOLI, 2023). Carbapenems are effective antimicrobials and are widely used for the treatment of infections caused by multidrug-resistant *Enterobacterales*. Due to the increase in its use, the great adaptive capacity of bacteria, which are able to develop or acquire different resistance mechanisms that allow them to survive exposure to antibiotics (REDDY et al., 2020; ZHANG et al., 2020). One of the main mechanisms of resistance to these antibiotics is the production of hydrolytic enzymes, among them the so-called carbapenemases (AURILIO et al., 2022). As an example, one can cite the *Klebsiella Pneumoniae* Carbapenemase (KPC) (FLORES et al., 2020), New Delhi metallo-β-lactamases (NDM) (YAO et al., 2022) and Oxacilinases (OXA) (RIVERA-IZQUIERDO et al., 2021).

CRKP carriers of carbapenemases-encoding genes are a cause for great concern, as their prevalence in nosocomial infections limits therapeutic options and is associated with high mortality rates (BUSH, 2018; VRANCANU et al., 2021). The increased prevalence of CRKP KPC and NDM enzymes in hospitals has already been reported in Brazil (VIVAS et al., 2020; WINK et al., 2021), especially during the period of the COVID-19 pandemic, due to the increased use of antimicrobials (POLLY et al., 2022).

Several reports have addressed the genetic relationship of CRKP associated with epidemiological data based on Multi-locus Sequence Typing (MLST) (FLORES et al., 2020; ANANTHARAJAH et al., 2022; ENANY et al., 2022; LORENZIN et al., 2022). It is a highly discriminative sequence-based typing method developed in the late 1990s for molecular epidemiological analysis of variety of bacterial pathogens (SPRATT, 1999; ENANY et al., 2022). Many Sequence Types (STs) are specific to certain geographic areas, and some are epidemic and/or endemic. For example, ST11 is more widespread in Asia (XIE et al., 2021; DUAN et al., 2022) and in South America (NICOLA et al.,...
This ST is a clone of great clinical importance because it is often associated with several virulence and resistance genes, mainly with the dissemination of \( \text{bla}_{\text{KPC}} \) (AZEVEDO et al., 2019; ANDREY et al., 2020) and \( \text{bla}_{\text{NDM}} \) (CAMARGO et al., 2022; NOVAIS et al., 2022).

An important source of dissemination of CRKP clones, outside the hospital environment, is the effluents from wastewater treatment plants (WWTP) (SUZUKI et al., 2020). These resistant pathogens are eliminated by human excreta and are transported to the WWTPs. Most of these stations do not have an efficient treatment system for the disinfection of effluents that are released directly into receiving water bodies (RIZZO et al., 2013).

CRKP strains belonging to clones considered of high clinical risk harboring resistance genes have already been found in aquatic environments such as recreational waters (CAMPANA et al., 2017), rivers (OLIVEIRA et al., 2014; NASCIMENTO et al., 2017) and wastewater (DROPA et al., 2016; MARTI et al., 2017; LEPUSCHITZ et al., 2019; POPA et al., 2021). The spread of CRKP strains carrying resistance genes outside the hospital environment through urban and hospital wastewater is a global public health concern (WAŚKO et al., 2022).

It is important to emphasize that clinical clones of CRKP such as ST11 (ZHANG et al., 2021; LÁZARO-PERONA et al., 2022), ST37 (MARKOVSKA et al., 2021; PICCIRILLI et al., 2021) and ST147 (LAPP et al., 2021; GONZALES-ESCALANTE et al., 2022; MATASEJE et al., 2022) have been also described in wastewater (OBASI et al., 2017; NÜESCH-INDERBINEN et al., 2018; SEKIZUKA et al., 2018), which indicates the importance of studies about their dissemination through environmental matrices (TEBAN-MAN et al., 2021).

According to One Health approach, efforts towards epidemiological surveillance of clinically important CRKP clones in WWTP wastewater are essential. Data on the circulation of multidrug-resistant clones are also relevant and capable of supporting
actions by decision makers regarding possible negative impacts to human and environmental health.

The objectives of this study were to characterize CRKP recovered from Untreated Wastewater (UWW) and Treated Wastewater (TWW) from three WWTPs (municipal, industrial and hospital) regarding their clonal relationships associated to antimicrobial resistance and carbapenemase-encoding genes profiles.

2. Materials and methods

2.1. Study setting and wastewater sampling

Three WWTPs, located in the city of Rio de Janeiro-Brazil, were selected for this study. WWTP1 is a municipal station that receives domestic and industrial wastewater and serves 30 neighborhoods in the city of Rio de Janeiro, with projected average flow of 2.5 m$^3$/s (220 L/s) and features a secondary treatment system with activated sludge. WWTP2 is a station that receives mixed/industrial wastewater, with the capacity to supply the sanitary sewage of an average population of 12,000 people and with an average projected flow of 512 m$^3$/day (5.92 L/s) and presents a secondary level biological treatment system with the activated sludge process, long-acting variant. WWTP3 treats effluent from a hospital center with 200 beds, in addition to the administrative and infrastructure areas, with an average projected flow of 65 m$^3$/day (0.75 L/s). It features a biological treatment system with the Moving Bed Biofilm Reactors (MBBR) process, followed by disinfection with sodium hypochlorite. The samples collection of UWW and TWW (1L of each) was carried out in October 2021 at the three WWTPs, using sterile containers. The samples were transported to the laboratory under refrigeration and were processed within 24 hours.

2.2. Isolation, identification, and antimicrobial susceptibility

For each sample, 50 mL of the UWW and 100 mL of the TWW were concentrated by filtration through a 0.22 µm nitrocellulose membrane. For the isolation of
carbapenem-resistant strains, the membranes were inoculated into tubes containing Brain Heart Infusion (BHI) broth supplemented with ceftazidime (4 mg L\(^{-1}\)) and ertapenem (0.5 mg L\(^{-1}\)) (EUCAST, 2022), and incubated at 37 °C for 24 hours. Then, the broth was seeded on Eosin Methylene Blue (EMB) agar and incubated at 37°C for 24 hours (LAL; CHEEPTHAM, 2007). All colonies with different morphologies were seeded on BHI agar, incubated at 37°C for 24 hours. After growth, the colonies were cryopreserved in BHI broth with 20% glycerol at -80°C (GERNA; GERHARDT, 1981).

The isolates were identified by protein profile using the Matrix Associated Laser Desorption-Ionization – Time of Flight Mass Spectrometry (MALDI-TOF/MS). (BENAGLI et al., 2011). After identification, the isolates were deposited in the “Coleção de Culturas de Vigilância em Saúde Única” (CCVSU) of “Instituto Nacional de Controle de Qualidade em Saúde” (INCQS)/FIOCRUZ with the following identifications: CCVSU 6996; CCVSU 7024; CCVSU 7028; CCVSU 7031; CCVSU 7047; CCVSU 7048; CCVSU 7051; CCVSU 7055; CCVSU 7058; CCVSU 7060; CCVSU 7084; CCVSU 7102; CCVSU 7110; CCVSU 7112; CCVSU 7113; CCVSU 7173; CCVSU 7174; CCVSU 7127.

The susceptibility of 11 antimicrobials from different classes, was performed by disc diffusion. The following antibiotics were tested: amikacin (30 μg), aztreonam (30 μg), cefepime (30 μg), ceftazidime (10 μg), ciprofloxacin (5 μg), ertapenem (10 μg), imipenem (10 μg), meropenem (10 μg), piperacillin/tazobactam (36 μg), tetracycline (30 μg), and sulfamethoxazole/trimethoprim (25 μg). As test control, strains Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 35218 were used.

The diameters of the inhibition halos were determined and evaluated according to the EUCAST (2022) protocols and criteria. The isolates were classified as Multidrug-resistant (MDR), Extensively drug-resistant (XDR), according to MAGIORAKOS et al., 2012. The phenotypic test of carbapenemase activity was performed using the commercial kit CARBAPENEMBAC® (PROBAC, Brasil) (MARTINO et al., 2015).
2.3. Detection of carbapenem-resistance genes

Genomic DNA was extracted using the PureLink Genomic DNA kit (Invitrogen). The isolates identified as *Klebsiella pneumoniae* were screened for the presence of the *bla*KPC, *bla*NDM, *bla*OXA-48-like, *bla*IMP, *bla*VIM, *bla*GES (MONTEIRO et al., 2012) and *bla*BKC (NICOLETTI et al., 2015) genes, whose amplification was performed by real-time PCR/High-Resolution Melting (HRM). The final PCR volume was 10 μl, including 10 μM of each specific primer, 2X MeltDoctor HRM Mastermix (Applied Biosystems) and thermal cycling profile according to MONTEIRO et al., 2012 and NICOLETTI et al., 2015. The DNA of the strains *K. pneumoniae* ATCC BAA1705 (*bla*KPC), *K. pneumoniae* CCBH 24217 (*bla*NDM), *K. pneumoniae* CCBH 24264 (*bla*OXA-48-like), *P. aeruginosa* CCBH 20008 (*bla*VIM), *P. aeruginosa* CCBH 24208 (*bla*IMP), *E. coli* BL21-pET-26BKC-1 (*bla*BKC), *P. aeruginosa* CCVSU 4479 (*bla*GES) were used as positive controls and *E. coli* ATCC 25922 was used as negative control.

The *bla*OXA-48-like was sequenced with fluorescent terminators (BigDye; Applied Biosystems, Foster City, CA) in SeqStudio (Thermofisher). Sequencing Analysis Software v7.0 (Thermofisher) was used to assess the quality of the generated readings. Ambiguous nucleotides were removed based on the quality score and sequences with a score less than 30 were removed. The assembly of high-quality sequences was performed in the BioEdit software. Sequences were analyzed using blastx in the CARD (Comprehensive Antibiotic Resistance Database) (ALCOCK et al., 2023).

Based on the carbapenem resistance genes researched, the isolates were classified into five carbapenemase genetic profiles.

2.4. *Klebsiella pneumoniae* Multilocus Sequence Type (MLST)

For the MLST of *K. pneumoniae* isolates, internal fragments of seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB* and *tonB*) were amplified by PCR, according
to DIANCOURT et al., 2005, and sequenced with fluorescent terminators (BigDye; Applied Biosystems, Foster City, CA) in SeqStudio (Thermofisher). Sequencing Analysis Software v7.0 (Thermofisher) was used to assess the quality of the generated readings. Ambiguous nucleotides were removed based on the quality score and sequences with a score less than 30 were removed. The assembly of high-quality sequences was performed in the BioEdit software. Sequences were deposited in the Pasteur Institute database MLST database (http://bigsdb.pasteur.fr/klebsiella/) for determination of Sequence Types (STs) and clonal complexes (CCs).

3. Results

3.1. Antimicrobial susceptibility, resistance genes and MLST profiles

Ninety-eight Enterobacterales were recovered, among these, 18 (18.4%) were identified as Klebsiella pneumoniae. These strains were isolated from the three WWTPs studied, seven from WWTP1 (2 UWW and 5 TWW), one from WWTP2 (UWW) and ten was recovered from WWTP3 (UWW) (Table 1).

All isolates presented higher percentages (more than 50%) of antimicrobials resistance in UWW, except for tetracycline, which presented the highest percentage (83%) of resistant in TWW (Fig. 1). Regarding resistance to carbapenems, 100% were resistant to ertapenem, with 28% being isolated from treated effluents. Seventy-two percent of the isolates were resistant to both imipenem and meropenem. However, while 8% of these imipenem resistant isolates were obtained from TWW samples, no meropenem resistant isolates were obtained from TWW. Among the resistance profiles, 38.9% (7/18) presented the MDR profile and 61.1% (11/18) presented the XDR profile. The phenotypic test of carbapenemase-producing showed 83.3% (15/18) of isolates were positive.
The isolates were classified in carbapenemase genetic profiles, 38.9% (7/18) profile I (blaKPC); 5.5% (1/18) profile II (blaNDM); 22.2% (4/18) profile III (blaKPC/blaNDM); 11.1% (2/18) profile IV (blaKPC/blaNDM/blaOXA-37); and 22.2% (4/18) profile V (no gene detected). The blaIMP, blaVIM, blaGES and blaBKC genes were not detected.

The clonal relationship of *K. pneumoniae* strains identified five different STs (ST11, ST37, ST147, ST244 and ST281. Among the STs, an isolate CCVSU 6996 belonging to ST37 was recovered from the UWW of WWTP2, the only isolate recovered from this station. Nine isolates, CCVSU 7028, 7031, 7047, 7048, 7051, 7055, 7058, 7060 and 7084, belonging to ST244, were recovered from the UWW of WWTP3 and no isolate was recovered from the TWW. Six isolates belonging to ST11, were recovered from WWTP1, CCVSU 7102 isolated from the UWW and five isolates, CCVSU 7110, 7112, 7113, 7173 and 7174 from the TWW. The strain CCVSU 7127, belonging to ST147, was isolated from the UWW of WWTP1. The strain CCVSU 7024, belonging to ST281, recovered from the UWW of WWTP3.

A Minimal Spanning Tree (MST) was created with the five STs found in this study and those deposited in the Pasteur Institute database according to the sources of isolation of the strains (Fig. 2).

### 4. Discussion

This study was carried out to investigate the occurrence and persistence of different clones of CRKP in UWW and TWW from three WWTPs located in the city of Rio de Janeiro, Brazil. Our results showed strains with MDR and XDR resistance profiles in UWW and TWW, and all isolates were resistant to at least one carbapenem. ZAGUI et al., 2021, found 62% MDR Gram-negative from treated hospital wastewater and all bacteria were resistant to at least one antimicrobial agent. Likewise, a study in Romania showed five CRKP resistant to at least one carbapenem, three from UWW and two from TWW with chlorine disinfection (POPA et al., 2021), differently of our study, which recovered CRKP only from secondary treatment plant. The detection of
these bacterial resistance profiles, as well as antimicrobial resistance genes could be considered a good monitoring system using wastewater-based surveillance (WHO, 2020).

The carbapenemase-encoding genes investigated in our study $\text{bla}_{\text{KPC}}, \text{bla}_{\text{NDM}}$, and $\text{bla}_{\text{OXA-370}}$ were recovered from both UWW and TWW. However, it is worth noting that $\text{bla}_{\text{KPC}}, \text{bla}_{\text{NDM}}$ were detected even after treatment as a means of spread with gradual health risk. These genes have been reported from several environmental sources in Brazil. For example, the recovery of CRKP $\text{bla}_{\text{KPC}}$ from hospital TWW (CHAGAS et al., 2011; PICÃO et al., 2013) and the presence of 65% of CRKP $\text{bla}_{\text{KPC}}$ and $\text{bla}_{\text{NDM}}$ only in treated sewage (PEREIRA et al., 2022). In the United States of America, LOUDERMILK et al., 2022, recovered higher relative abundance of $\text{bla}_{\text{KPC}}$ (1.25 copies/16S rRNA) only in the aeration tank stage of a treatment station. On the other hand, in our study, this gene was revealed both in UWW and TWW. Another study carried out also found the $\text{bla}_{\text{NDM}}$ gene in greater abundance in Klebsiella sp. recovered of municipal wastewater nitrification tanks in Czech Republic (STACHUROVÁ et al., 2021). The $\text{bla}_{\text{OXA-48-like}}$ gene variant, $\text{bla}_{\text{OXA-370}}$, has been previously reported in the clinical setting in Brazil, co-carried with $\text{bla}_{\text{NDM}}$ in Enterobacter spp. and Klebsiella pneumoniae strains (BOYD et al., 2022).

In our study, among isolates containing carbapenemase-encoding genes, 42.8% co-carried at least two resistance genes simultaneously. These strains tend to be extremely high resistant leading to limitations in the clinical treatment (YANG et al., 2022). Multiple resistance genes are commonly co-carried by plasmids, which are mobile genetic elements that facilitate the horizontal transference genes among the bacterial community (LERMINIAUX; CAMERON, 2019). GAO et al., 2020, recovered four CRKP isolates from patient specimens co-hosting $\text{bla}_{\text{KPC}}$ and $\text{bla}_{\text{NDM}}$ genes encoded on the same plasmid IncR+IncFII(pHN7A8), increasing the possibility resistance dissemination in the environment (EBOMAH; OKOH, 2020; TEBAN-MAN et al., 2022).
According to Klebsiella pneumoniae MLST database, until the moment of this study, among the STs found, ST11 is the most prevalent (n=3,128), followed by ST147 (n=1,899), ST37 (n=642), ST244 (n=34) and ST281 (n=28). All STs found in this study have already been reported in North America, South America, Europe (except ST244), Asia (except ST281) and Africa (except ST244 and ST281). It is important to emphasize that the 5 STs found in our study were isolated from clinical samples and had already been described in Brazil. The Minimum Spanning Tree analysis (MST) (Fig. 2) shows the allocation of the strains analyzed in this study according to their STs. The prevalent ST11 has been associated with clinical strains and increasing antibiotic resistance showing MDR and XDR profiles. The MST analysis shows ST11 as the central ST from where other related ST are originated. In the MST tree, besides ST11, the other four ST here described, show different levels of relation to ST11. The ST with the closest relation to ST11 is ST244 sharing four alleles. The remaining ST37, ST147 and ST281 share only two alleles with ST11. This observation strongly suggests that ST244 could be grouped in a ST11 clonal complex (CC11). It is important to highlight that among all ST11 strains deposited in the database, only 5% were isolated from an environmental source, while for ST244 the percentage of environmental strains is 26.5% showing that ST11 is clearly non-environmental related. Besides the genetic relationship between ST11 and ST244, these two ST shares other important features related to antimicrobial resistance. Half (50%) of the ST11 strains isolated in this study show a XDR profile and KPC, NDM and OXA-370 producing, while 77% of the ST244 strains are XDR, also producing those enzymes. Because of this antimicrobial resistance profile among ST11 and ST244 strains, the suggested CC11 harbors 66.6% of XDR strains, which is a relevant feature to be considered when strains related to this CC are isolated. These data suggest that despite the large number of clinical strains classified as ST11, antimicrobial resistance related genes are much present among ST244 strains. In addition, it is important to emphasize that this is the first study reporting ST244 strains of wastewater.
In our study, nine CRKP ST244 strains isolated from the UWW of WWTP3 (hospital) were prevalent. One of them, CCVS 7048 XDR, presented genetic profile IV, with three genes simultaneously ($\text{bla}_{\text{KPC}}$, $\text{bla}_{\text{NDM}}$ and $\text{bla}_{\text{OXA-370}}$) and two genes profile III, CCVS 7060 XDR and CCVS7084 MDR ($\text{bla}_{\text{KPC}}$ and $\text{bla}_{\text{NDM}}$). Only strain CCVS 7051 from ST244 showed carbapenemase activity, however, none of the investigated resistance genes were detected. Thus, this strain may carry other genes not researched in this study. Few studies reported CRKP ST244, for example, fecal-detection VIM-producing $K.\ pneumoniae$ strain from Spain (ÖSTERBLAD et al., 2012), another study also reported KPC-producing CRKP from a single US institution (MATHERS et al., 2017).

The CCVSU 7024 XDR ST281 strain isolated from UWW (WWTP3) did not show any of the seven carbapenemase-coding genes nor carbapenemase activity in this study. However, it showed phenotypic resistance to the three tested carbapenems, signaling the presence of another resistance mechanism, such as loss of its main porins (OmpK35 and OmpK36), thus decreasing cell wall permeability and overexpression of the AcrAB efflux pump (DALMOLIN et al., 2017). Some studies demonstrated CRKP ST281 from clinical samples with a hypermucoviscous phenotype in Mexico and from two strains carrying the $\text{bla}_{\text{NDM}}$ gene in Brazil (GARZA-RAMOS et al., 2018; CAMARGO et al., 2022). In any case, to the best of our knowledge, this is the first report of strain ST281 recovered from wastewater.

At WWTP3, ten CRKP strains were isolated from UWW, and no strains were recovered from TWW. This fact demonstrates that the tertiary treatment with sodium hypochlorite present in this WWTP could be efficient in removing CRKP in wastewater. Sodium hypochlorite is widely used in wastewater disinfection to control the spread of antimicrobial resistant bacteria into the environment (BONETTA et al., 2021). Other studies have already demonstrated the efficiency of this treatment for resistant bacteria (TUROLLA et al., 2018; HASSABALLAH et al., 2019). Untreated and treated effluent from WWTP2 demonstrated the presence of several $Enterobacterales$ species other
than CRKP. However, a CRKP ST37 strain was revealed on the UWW. This strain ST37 are reported as potentially high-risk MDR (CHEN et al., 2021) and has been reported worldwide, such as three strains isolated from long-term care facilities, carrying the \textit{bla}_{KPC-2} gene inserted in plasmids IncFII(K), IncFIB(K) and IncN in Italy (PICCIRILLI et al., 2021); eleven strains from pediatric patients, carrying \textit{bla}_{NDM1} gene in China (TIAN et al., 2018); one strain carrying the \textit{bla}_{OXA-48} gene inserted in the IncL/M plasmid in Croatia (JELIĆ et al., 2018) and recently one strain isolated from public hospitals carrying the \textit{bla}_{KPC} gene, in Brazil (RODRIGUES et al., 2022). Furthermore, CRKP ST37 was recovered in wastewater from pharmaceutical industries carrying only ESBL genes in Nigeria (OBASI et al., 2017).

Another ST found in our study was ST147 MDR harbouring \textit{bla}_{KPC} and \textit{bla}_{NDM} simultaneously from UWW of WWTP1. This ST147 is considered a high-risk multidrug-resistant clone with worldwide nosocomial outbreaks and epidemic potential (PEIRANO et al., 2020; ARCARI; CARATTOLI, 2023). It has already been reported in hospital wastewater treatment plants, with an MDR profile, carrying the \textit{bla}_{NDM-1} gene inserted in the IncR plasmid in Germany (KEHL et al., 2022), \textit{bla}_{OXA-48} in Romania (TEBAN-MAN et al., 2021), \textit{bla}_{NDM-9} inserted into the IncR plasmid in Switzerland (NÜESCH-INDERBINEN et al., 2018) and \textit{bla}_{NDM-1} inserted into the plasmids IncA/C and IncX3 in the Philippines (SUZUKI et al., 2020). Recently, OLIVEIRA et al., 2023 also recovered two strains CRKP ST147 from UWW of WWTP, harboring \textit{bla}_{KPC-2} and \textit{bla}_{NDM-1} each one, in Brazil.

In our study, ST11 was the only ST that presented isolates both in UWW and TWW of the same WWTP. It is an important high-risk global multidrug-resistant clone of great clinical concern, as it presents hypermucoviscosity, accumulates multiple genes in plasmids, mainly \textit{bla}_{KPC-2} genes (LIAO; LIU; ZHANG, 2020), and contribute significantly to the spread of carbapenemase-encoding genes (CHEN et al., 2022). These characteristics turn the treatment and control of CRKP infections difficult, being the cause of serious clinical infections, representing a major global public health problem
nowadays (ZENG et al., 2022). In Brazil, most CRKP strains carrying the \textit{bla}_{KPC} gene in clinical samples belong to ST11 (CONCEIÇÃO-NETO et al., 2022). For example, recent reports of CRKP ST11 \textit{bla}_{KPC-2} and \textit{bla}_{NDM-7} inserted into an IncX3 plasmid in Intensive Care Units (CAMARGO et al., 2022; GASPAR et al., 2022).

Beyond clinical setting, CRKP ST11 have also already been isolated from environmental sources such as effluents WWTP in different parts of the world like Japan (SEKIZUKA et al., 2018); Austria (LEPUSCHITZ et al., 2019); in Ireland from hospital effluents, carrying the genes \textit{bla}_{OXA-48} and \textit{bla}_{KPC-2} (HOOBAN et al., 2021) and in effluent in Uruguay, carrying the \textit{bla}_{KPC} gene in plasmid pKPC-146 (SALAZAR et al., 2021). In Brazil, \textit{K. pneumoniae} ST11 strains have previously been reported in environmental matrices such as urban lakes and mangrove waters carrying the \textit{bla}_{KPC-2} gene inserted in the IncN plasmid (NASCIMENTO et al., 2017; CALISTRI et al., 2022) and in recreational coastal waters carrying the \textit{bla}_{NDM} gene (CAMPANA et al., 2017). Recently, OLIVEIRA et al. (2023) recovered thirteen CRKP ST11 strains from UWW and TWW in the same WWTP from Brasilia (Brazil), co-harboring \textit{bla}_{KPC-2} and \textit{bla}_{NDM-1} genes. This data corroborates the findings of this study, evidencing the survival potential of this concerning clone to the conventional treatment of the wastewaters.

In this study, CRKP ST11 strains were isolated in the UWW and TWW of a large municipal WWTP in the city of Rio de Janeiro, this result highlights the ability of CRKP ST11 to survive secondary treatment and suggests the possible contribution of this WWTP to the dissemination of multidrug-resistant pathogenic strains for the environment. It’s noteworthy that this clone was isolated from UWW carrying three genes simultaneously and in the TWW there are isolates presenting two of these genes or none of them, which suggest the loss of these genes during the treatment. The fact that more isolates were recovered in the TWW (5) than in the UWW (1), may represent that CRKP strains are increasing throughout the WWTP treatment, suggesting the low efficiency of secondary treatment level in removing these types of strains, and can be
contributing to the environmental dissemination of pathogenic strains that could transport carbapenemase genes.

It is noteworthy that CRKP belong STs 244 and 281 of environmental source were described for the first time, according to the MLST database. This demonstrates the relevance of clonal relationship studies using the MLST of clinically important multidrug-resistant strains isolated from WWTPs for environmental surveillance by assessing the status of these strains circulating in the country, in addition to confirming the contribution of WWTPs in the environmental dissemination of these strains, which represents a great risk to human and environmental public health.

Our results revealed the occurrence of multidrug-resistant CRKP clones simultaneously carrying one or more high clinical risk genes (bla<sub>KPC</sub>, bla<sub>NDM</sub> and bla<sub>OXA-370</sub>) in untreated and treated wastewaters from municipal wastewater treatment plants, can cause negative impacts on the environment and human health. These results contribute to a better understanding of the environmental dissemination of CRKP and its resistance genes, which is a relevant public health problem in the Brazilian epidemiological context and, at the same time, highlight the need for the implementation of more efficient treatment of effluents from WWTPs, in order to mitigate their risks in the dissemination of bacterial resistance in the aquatic environment.

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Conflict of Interest

No conflict of interest declared.

Data Availability

All the sequences generated in this study were deposited in the Institute Pasteur MLST/BIGSdb database.

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Authors contribution statement
KSM collected the samples, performed experimental works and data analysis, wrote the manuscript; CF performed experimental works and data analysis; APAN performed experimental works and data analysis; ASGB performed experimental works and data analysis; MM contributed to the data analysis and the writing of the paper; AG performed experimental works and data analysis; IF, MMC, KB contributed to the experimental work, data analysis, and the writing of the paper (review and editing); EMS and PRGB was responsible for funding acquisition, project administration, supervision, and contributed to the writing of the paper (review and editing). All authors read and approved the manuscript.
Figure 1. Antimicrobial resistance of Klebsiella pneumoniae with WWTP collection points. TWW: Treated Wastewater; UWW: Untreated Wastewater.
Figure 2. Minimal Spanning Tree analysis of all clinical and environmental Klebsiella pneumoniae isolated in Brazil deposited in the Pasteur Institute MLST database. The ST11, ST37, ST147, ST244 and ST281 described in this study are highlighted in red. Source of isolation is indicated by different colors.
<table>
<thead>
<tr>
<th>Strain</th>
<th>WWTP</th>
<th>Collection points</th>
<th>Resistance Phenotype</th>
<th>Resistance Profile</th>
<th>Carbapenemase Activity</th>
<th>Carbanemase Genes</th>
<th>ST</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCVSU 6996</td>
<td>2</td>
<td>UWW</td>
<td>SXT, AK, MRP, ERT, FEP, PIT, ATM</td>
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<td>blaKPC</td>
<td>I</td>
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<td>Negative</td>
<td>ND</td>
<td>V</td>
<td>281</td>
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<tr>
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<td>XDR</td>
<td>Positive</td>
<td>blaKPC</td>
<td>I</td>
<td>244</td>
</tr>
<tr>
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<td>SXT, CIP, AK, IMI, MRP, ERT, FEP, PIT, ATM</td>
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<td>blaKPC</td>
<td>I</td>
<td>244</td>
</tr>
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<td>Positive</td>
<td>blaKPC/blaNDM/blaOXA-370</td>
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<td>244</td>
</tr>
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<td>SXT, CIP, AK, IMI, MRP, ERT, FEP, CAZ, PIT, ATM</td>
<td>XDR</td>
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<td>blaKPC</td>
<td>I</td>
<td>244</td>
</tr>
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<td>CCVSU 7051</td>
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<td>UWW</td>
<td>SXT, CIP, AK, IMI, MRP, ERT, FEP, CAZ, PIT, ATM</td>
<td>XDR</td>
<td>Positive</td>
<td>blaKPC</td>
<td>I</td>
<td>244</td>
</tr>
<tr>
<td>CCVSU 7055</td>
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<td>UWW</td>
<td>SXT, CIP, AK, IMI, MRP, ERT, FEP, CAZ, PIT, ATM</td>
<td>XDR</td>
<td>Positive</td>
<td>blaKPC</td>
<td>I</td>
<td>244</td>
</tr>
<tr>
<td>CCVSU 7058</td>
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<td>SXT, AK, IMI, MRP, ERT, FEP, PIT, ATM</td>
<td>MDR</td>
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<td>blaKPC</td>
<td>I</td>
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</tr>
<tr>
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<td>blaKPC/blaNDM</td>
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<td>ND</td>
<td>V</td>
<td>11</td>
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<td>Negative</td>
<td>ND</td>
<td>V</td>
<td>11</td>
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<td>blaNDM</td>
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<td>11</td>
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<td>blaKPC</td>
<td>I</td>
<td>11</td>
</tr>
<tr>
<td>CCVSU 7174</td>
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<td>TWW</td>
<td>SXT, CIP, IMI, MRP, ERT, FEP, CAZ, PIT, ATM</td>
<td>XDR</td>
<td>Positive</td>
<td>blaKPC/blaNDM</td>
<td>III</td>
<td>11</td>
</tr>
<tr>
<td>CCVSU 7127</td>
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<td>UWW</td>
<td>TE, CIP, ERT, FEP, CAZ, ATM</td>
<td>MDR</td>
<td>Positive</td>
<td>bla\textsubscript{KPC}/bla\textsubscript{NDM}</td>
<td>III</td>
<td>147</td>
</tr>
<tr>
<td>-------------</td>
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<td>------</td>
<td>-----------------------------</td>
<td>-----</td>
<td>----------</td>
<td>-----------------</td>
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<td>-----</td>
</tr>
</tbody>
</table>

AK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CCVSU, “Colecção de Culturas de Vigilância em Saúde Única”; ERT, ertapenem; FEP, cefepime; IMI, imipenem; MDR, multidrug-resistant; MRP, meropenem; ND, no gene detected; PIT, piperacillin/tazobactam; ST, sequence type; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; TWW, treated effluent; UG, ungrouped; UWW, untreated wastewater; WWTP, wastewater treatment plant XDR, extensively drug-resistant.

**Table 1.** Characterization of carbapenem-resistant *Klebsiella pneumoniae* strains.