*Original Research Article*

Detection of KPC-Producing Enterobacteria and Antimicrobial Resistance in Brazilian Sewage Treatment Plants

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ABSTRACT

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| **Background and Aims:** Pathogenic microorganisms can be found in sewage and may cause several infections and deleterious health effects. In this scenario, Enterobacterales can trigger several diseases in humans and animals, since antimicrobial resistance bacteria, as well as antimicrobial resistance genes, are considered a global public health problem that tends to increase. Therefore, this study aims to identify Enterobacterales resistant to third- and fourth-generation cephalosporins and carbapenems isolated from Sewage Treatment Plants (STP) in Brazil.  **Methodology:** Four sampling campaigns were performed weekly from the affluent and effluent of two STPs at the Oswaldo Cruz Foundation. In each campaign, four samples were collected in different points in the affluent at the entrance to the STP and the effluent at the exit. A chromogenic medium infused with third-generation cephalosporin was employed, which Gram-negative and oxidase-negative bacteria underwent conventional screening and biochemical tests. Identification was performed with an automated method in VITEK 2® (BioMérieux). The antimicrobial susceptibility tests (AST) and phenotypic assays for carbapenemase detection were evaluated using the agar diffusion methodology in Müeller-Hinton agar, and antibiotic disks suitable for *Enterobacterales.* The profile of enterobacteria was categorized as Sensitive (S), Intermediate (I), and Resistant (R). The identification of genes related to bacterial resistance were based on PCR reactions, where DNA was extracted using the heat shock method.  **Results:** Eight species were identified in the 19 Enterobacterales isolates. Of these, 26% were *Klebsiella pneumoniae*, 16% *Escherichia coli,* 16% *Citrobacter freundii*, 16% *Kluyvera cryocrescens*, 11% *Kluyvera intermedia*, 5% *Enterobacter asburiae*, 5% *Citrobacter farmeri* and 5% *Citrobacter amalonaticus.* *K. pneumoniae* was isolated in all samples of raw sewage from STP B, which was already expected since STP B receives hospital waste. However, it was not found in STP A or any sample of treated sewage from either STP. Among these isolates, 75% were non-sensitive to ciprofloxacin, 71% to ceftriaxone, 57% to ceftazidime, 46% to cefoxitin, 36% to cefepime, 28% to piperacillin + tazobactam, and 11% to gentamicin, respectively. Phenotypic tests effectively screened carbapenem resistance mechanisms, displaying high concordance with molecular tests. These findings indicate the presence of the blaKPC gene, reflecting the predominance of this enterobacteria in Brazil.  **Conclusion:** A high profile of non-sensitivity to most antimicrobials tested, especially third-generation cephalosporins, quinolones, and carbapenems were reported. These results emphasize the dissemination of potentially pathogenic and antimicrobial-resistant microorganisms in the environment from STP’s final effluents, regardless of the type of system adopted. Therefore, the concern related to environmental contamination and the spread of bacterial resistance to antimicrobials in aquatic reservoirs must be addressed. |

*Keywords: Enterobacterales; chromogenic; antimicrobials; bacterial pharmacoresistance.*

1. INTRODUCTION

Human activities generate waste in several processes that must be managed appropriately under established environmental guidelines. However, this management is not always adequately performed, leading to deleterious impacts on humans, animals, and environment, carrying several pathogenic agents [1]. Waste contamination-related diseases have emerged with technological advances and industrialization, and there is a need to reduce this degradation through strategies based on ecological and populational preservation of all exposed species, solving severe issues of scarce natural resources and increasing pollution [2]. To minimize the impact of human activity in the environment, sewage treatment plants (STPs) employ activated sludge processes where microorganisms degrade organics and remove nutrients from wastewater, producing quality effluent.

The primary modality of liquid waste treatment involves physically removing large and small particles through filtration and sedimentation. Although many pollutants remain in the effluents flowing from the STPs to natural water bodies nearby. In such cases, the microbial community in the activated sludge reactor (an artificial environment) deals with the first-hand pollution, while the microbial community in the effluent receiving area (a natural environment) suffers second-hand pollution [3]. Sewage can be classified as domestic, pluvial, or industrial. In Brazil, as in many other developing countries, many cities need improved sanitation procedures, while others do not have any at all [4]. Due to the contamination of sewage by several microorganisms, the risk of infections related to their presence in these reservoirs increases. Bacteria are responsible for most of these infections, and *Enterobacterales* bacteria are primarily responsible for several health problems [5,6].

Enterobacteria are Gram-negative bacilli belonging to the *Enterobacterales* order. Until recently, the *Enterobacteriaceae* family grouped practically all-important genera of enterobacteria. However, phylogenetic studies proposed new families, and currently, the order comprises seven families: *Enterobacteriaceae*, *Erwiniaceae*, *Morganellaceae*, *Yersiniaceae*, *Pectobacteriaceae*, *Hafniaceae*, and *Budviciaceae* [7], facultative anaerobic or aerobic, non-sporulating bacteria with variable motility, generally oxidase-negative, growing in basic, rich, or selective media. They can reduce nitrate to nitrite and ferment glucose by producing acid or acid and gas [8,9]. These microorganisms are widely distributed in nature. They are found in water, soils, plants, and human and animal intestinal tracts. They are the primary components of the gut microbiota but are relatively uncommon elsewhere in the body and may be responsible for several infectious processes [8,9].

Bacterial antimicrobial resistance is a cellular defense mechanism to preserve the microorganism’s survival, which can be intrinsic or acquired. Strains resistant to conventional antimicrobials reduce the likelihood of effective treatment of infectious diseases caused by such microorganisms [10,11]. Using chromogenic media to isolate Gram-negative bacilli helps to streamline the identification process of the *Enterobacterales* bacteria. These media with added antimicrobials are widely employed in clinical assays for the identification of these bacteria. Few environmental studies still employ chromogenic media as a routine as they have gradually gained space due to their satisfactory results [12].

This study aimed to identify *Enterobacterales* bacteria isolates in Sewage Treatment Plants (STP) in Rio de Janeiro – Brazil using chromogenic medium with third-generation cephalosporin. The importance of the study relies on the fact that wastewater plants in Rio de Janeiro release the effluent in the main rivers in the city, which the presence of enterobacteria could be deleterious for the health of the population inv thew surroundings. The medium selection was followed by the antimicrobial susceptibility test (AST) for complete isolation and identification. Phenotypic tests for the detection of carbapenemase and the research of genes related to bacterial resistance to third- and fourth-generation cephalosporins and carbapenems were also performed.

2. material and methods

Methodologies based on the American Public Health Association were applied for the sewage analysis [13]. Samples were processed in the Microbiology Laboratory (Labmicro) of the Department of Biological Sciences at Sérgio Arouca National School of Public Health – FIOCRUZ.

**2.1 Studied area and sampling collection points**

Two STPs at the Oswaldo Cruz Foundation were selected for this study and named A and B. Laboratory-borne effluents were treated as STP A and effluents from hospital as STP B, responsible for treating the effluent originated at the hospital built to address the COVID-19 pandemic in Rio de Janeiro. The STP A operates as a biological treatment system at a secondary level with the activated sludge process through prolonged aeration. The STP B includes a biological treatment system with the Moving Bed Biofilm Reactors (MBBR) process followed by sodium hypochlorite disinfection.

Four sampling campaigns were performed weekly from the affluent and effluent of each site to facilitate a better comparison between the results [13]. In each campaign, four samples were collected in different points in the affluent at the entrance to the STP and the effluent at the exit. All samples were collected in triplicate during the morning period between 9 and 11 am.

**2.2 Seeding and bacterial biochemical identification**

Sewage samples, after being received at the laboratory, were seeded directly into the culture media (100μL), and dilutions were also performed in sterile saline solution (0.9% NaCl), at concentrations of 10-1 to 10-4 for treated sewage and 10-1 to 10-6 for raw sewage. Then, 10μL of each dilution were seeded in a chromogenic culture medium containing antimicrobial cefotaxime of the third-generation cephalosporin class (2 mg/L), CHROMagar ESBL (Plast Labor®). Seeding was performed by draining the sample directly and enriching it in a Brain Heart Infusion Broth (BHI) medium [12-16]. Gram-negative and oxidase-negative bacteria underwent conventional screening and biochemical tests [8,9,17,18]. Gram-negative bacteria were subjected to Costa & Vernin screening media [17], SIM (Hydrogen Sulfide/Indole/Motility), Urea Base Agar (Christensen), Citrate Agar (Simmons), glucose fermentation with verification of gas production and deamination of the amino acids lysine, arginine and ornithine. The VP test (Voges-Proskauer) was performed to differentiate between *Aeromonas* sp. species. After seeding, these media were incubated in an oven at 35°C ± 1°C for 18 to 24 hours. Identification was performed in parallel in VITEK 2® (BioMérieux), an automatic photometric reading device, and the results were obtained after 5-6 hours.

**2.3 Antimicrobial Sensitivity Test**

The preparation of the bacterial inoculum was based on the Mc Farland turbidity scale (0.5 tube). Sensitivity of the isolated microorganisms was evaluated using the agar diffusion methodology (disk diffusion) in Müeller-Hinton agar, and antibiotic disks (Oxoid®) suitable for *Enterobacterales*, such as ampicillin, ampicillin+sulbactam, piperacillin+tazobactam, amoxicillin+clavulanic acid, cefoxitin, ceftriaxone, ceftazidime, cefepime, ciprofloxacin, gentamicin, amikacin, imipenem, ertapenem, meropenem and polymyxin B/colistin [16,19]. The plates were placed in the incubator at 35±1°C for 18±2 hours and the result of the agar diffusion test was obtained by measuring the diameter of the growth inhibition halo, where bacteria were categorized as Sensitive (S), Intermediate (I), and Resistant (R). The antimicrobial susceptibility test was performed based on the compendia accepted in the country: the Clinical and Laboratory Standards Institute (CLSI) and the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST), which is based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [16,19].

**2.4 Phenotypic tests**

The disk approximation test was employed for the phenotypic detection of ESBL production in enterobacteria, using amoxicillin with clavulanic acid, cefotaxime, ceftazidime, and cefepime [20]. Meropenem and cefoxitin disks were used for the AmpC-type β-lactamases detection test [16]. Bacterial strains that showed resistance or reduced sensitivity to carbapenems, according to CLSI recommendations [16], were submitted to the modified Hodge test using ertapenem, imipenem, or meropenem disks [21]. Ertapenem, meropenem, and imipenem disks, impregnated with and without phenylboronic acid (AFB), were used for the phenotypic detection of class A carbapenemases, mainly of the *Klebsiella pneumoniae* carbapenemase (KPC) subtype [23, 24]. In this test, phenylboronic acid performs the enzymatic blockade of this carbapenemase type [25].

Ethylenediaminetetraacetic acid (EDTA) can chelate zinc ions from the active site of metallo-beta-lactamases, such as New Delhi metallo-beta-lactamase (NDM). Cloxacillin (CLOXA) is used for the suggestive detection of plasmid Ampicillinase C (AmpC) to observe the potentiation when added to carbapenems [24,26]. For the experiments, the ANVISA protocols [25] were followed, and they indicate phenotypic screening of the abovementioned mechanisms. Extended Spectrum Beta-lactamases (ESBL) and AmpC tests were performed on all enterobacteria isolated from the chromogenic medium with cefotaxime, as resistance to some third generation cephalosporins indicates this. Hodge’s tests, with AFB, EDTA, and CLOXA, were performed on enterobacteria not sensitive to some carbapenems because it indicates a possible carbapenemase producer [16,25].

**2.5 Molecular tests**

DNA was extracted using the heat shock method from a recent culture originating from the nutrient agar medium [27-29]. The DNA was initially denatured by heating at 94° C for 5 minutes. Then, the material was subjected to 40 thermal cycles: 30 seconds at 94° C for denaturation, 30 seconds at 50° C for annealing, and 72° C for extension, with duration varying according to the target size, considering a ratio of 1 min/1 Kb. PCR reactions were performed to detect the gene sequences shown in Table 1. These sequences were chosen based on the country’s epidemiological profile [27, 30, 31]. Amplification by the PCR reaction was visualized in agarose gel (2%) [27,28,32]. The records were compared with the standard (AGARGEN®) and subsequently analyzed for the association of molecular and phenotypic findings.

**Table 1. Primers applied in PCR reactions for the identification of the presence of resistant genes.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Primers** | **Nucleotide sequence** | **Target region** | **Size** | **Reference** |
| KPC- F KPC- R | ATGTCACTGTATCGCCGTC TTACTGCCCGTTGACGCC | Alleles *bla*KPC | 882 bp | LGMM |
| IMP- F IMP- R | GAAGGCGTTTATGTTCATAC GTAAGTTTCAAGAGTGATGC | Alleles *bla*IMP | 587 bp | LGMM |
| VIM- F VIM- R | GTTTGGTCGCCATATCGCAAC AATGCGCAGCACCAGGATAG | Alleles *bla*VIM | 382 bp | LGMM |
| BKC- F BKC- R | ACATAATCTCGCAACGGGCG TCGCCGGTCTTGTTCATCAC | Alleles *bla*BKC | 512 bp | Nicoletti *et al*. (2015) |
| OXA-23- F OXA-23- R | GATGTGTCATAGTATTCGTCG TCACAACAACTAAAAAGCACTG | EAG 3` of *bla*OXA-23  EAG 5` of *bla*OXA-23 | 1064 bp | Wang; Zong; Lu (2011) |
| OXA-48- R OXA-48- F | GCGTTTTATGTCTAACAGTCC AAGTAGCATCAGTCCATCC | EAG 3` of *bla*OXA-48  EAG 5` of *bla*OXA-48 | 744 bp | LGMM |
| CTX-M- F CTX-M- R | TTAATGATGACTCAGCA GATACCTCGCTCCATTTAT | Alleles *bla*CTX-M | 800 bp | LGMM |
| F (Forward) / R (Reverse) / EAG – End Adjacent Genus / bp – base pairs/ LGMM – Laboratory of Molecular  Genetics of Microorganisms – Oswaldo Cruz Institute (IOC-FIOCRUZ). *The authors (2024).* | | | | |

3. results and discussion

**3.1 Qualitative analysis of identified bacteria**

Eight species were identified among the nineteen *Enterobacterales* isolates. Of these, 26% were *Klebsiella pneumoniae* (5), 16% *Escherichia coli* (3), 16% *Citrobacter freundii* (3), 16% *Kluyvera cryocrescens* (3), 11% *Kluyvera intermedia* (2), 5% *Enterobacter asburiae* (1), 5% *Citrobacter farmeri* (1), and 5% *Citrobacter amalonaticus* (1) of the total number in this studied group. The *K. pneumoniae* specie was isolated in all samples of raw sewage from the STP B. However, it was not found in STP A or any sample of treated sewage from either STP. The presence of this specie in non-treated sewage was already expected since the STP B receives hospital waste [4, 31, 33-35]. A high detection frequency of *K. pneumoniae* is noted in health care institutions and their waste [6,12,14,20].

*E. coli* was found in the treated wastewater in the second sample from the STP A. Puljko *et al.* previously detected *E. coli* in municipal STP in a Croatian study, even after wastewater treatment [39]. *E. coli* is one of the most frequent bacteria in wastewater and is easily found in domestic sewage. As previously mentioned, the importance of this species makes it the object of research in several reservoirs, food, and water for human consumption [2, 36-38].

Three representative species of *Citrobacter sp.* were found in the samples namely *C. freundii, C. farmeri, and C. amalonaticus*. The genus was found in both STPs, even in treated wastewater. Facciolà et al. described the presence of *Citrobacter sp.* in the sewage of slaughterhouses in Sicily (Italy) as 7.5% of the total bacteria surveyed. In comparison, the genus represented 9% of the total surveyed in the present study [38]. *Citrobacter sp.* is a genus of clinical importance since it belongs to the group of coliforms researched mainly for water and food quality [40, 41]. It can also cause several infections, including Healthcare-Associated Infections (HAI), as shown in the studies on urinary infection and the research with isolates from tracheal secretion and oral cavity [42,43].

*Kluyvera cryocrescens* and *K. intermedia* accounted for 27% of the enterobacteria findings. *Kluyvera sp.* is not frequently evaluated in sewage and wastewater studies as the species previously mentioned. However, it is equally significant concerning pathogenicity. In China, carbapenem-resistant genera in isolated *K. cryocrescens* strains were detected from hospital sewage [44]. *Kluyvera sp.* was isolated from a human gastrointestinal tract infection, showing that bacteria of this genus can, like any other enterobacteria, cause several infections [45]. Hernández-García et al. isolated six *Kluyvera sp.* hospital strains encompassing antimicrobial-resistance genera [46]. This study showed that even lesser-known species can be reservoirs of resistant genera, increasing the risk of their transmission to other species or genera [46].

Among enterobacteria, the last isolated representative was the genus *Enterobacter*, detecting *E. asburiae* in only one raw sewage sample at the STP A. This genus is similarly related to *Klebsiella sp.* regarding HAIs and bacterial resistance to antimicrobials [12, 48, 48]. Wu et al. detected *Enterobacter sp.* in blood samples from patients with sepsis [50]. Baker and Gardner reported a skin lesion concomitantly infected by *E. asburiae* and *Aeromonas hydrophila*. These two microorganisms can act synergistically with other bacteria in developing and aggravating infections [51].

**3.2 Antimicrobial sensitivity profile**

The profile of enterobacteria was categorized as Sensitive (S), Intermediate (I), and Resistant (R) as shown in Table 2. Bacteria submitted to the AST were previously screened using a chromogenic medium with cefotaxime. The term non-sensitive is used to designate categories I and R together [16].

**Table 2. Profile of isolated enterobacteria’s sensitivity to antimicrobials**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **ID** | **Species** | **AMP** | **ASB** | **PIT** | **CFO** | **CAZ** | **CPM** | **CRO** | **ETP** | **IMI** | **MEM** | **AMI** | **GEN** | **CIP** | **COL** |
| 1EBC1 | *Escherichia coli* | R | R | R | S | R | I | R | S | S | S | S | S | R | S |
| 1EBINI2 | *Klebsiella pneumoniae* | R | R | R | R | R | R | R | R | R | R | R | S | R | S |
| 1EBINI3 | *Klebsiella pneumoniae* | R | R | R | R | R | R | R | R | R | R | R | S | R | S |
| 2EBINI3 | *Citrobacter farmeri* | R | R | R | R | R | R | R | R | R | R | S | S | R | S |
| 2EBINI4 | *Klebsiella pneumoniae* | R | R | R | R | R | R | R | R | R | R | R | S | R | S |
| 2ETC1 | *Escherichia coli* | R | S | S | S | R | I | R | S | S | S | S | S | R | S |
| 2ETC4 | *Kluyvera cryocrescens* | R | S | S | R | S | S | R | S | S | S | S | S | R | S |
| 2ETC5 | *Kluyvera cryocrescens* | R | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 2ETINI1 | *Kluyvera cryocrescens* | R | S | S | S | S | S | R | S | S | S | S | S | S | S |
| 3EBC2 | *Enterobacter asburiae* | R | R | I | R | R | S | R | S | S | S | S | S | R | S |
| 3EBC3 | *Citrobacter freundii* | R | R | S | R | I | I | R | S | S | S | S | S | R | S |
| 3EBC4 | *Escherichia coli* | R | I | S | S | S | S | R | S | S | S | S | S | S | S |
| 3EBINI1 | *Klebsiella pneumoniae* | R | R | R | S | R | R | R | R | R | R | R | S | R | S |
| 3ETC2 | *Citrobacter freundii* | R | R | S | R | I | I | R | S | S | S | S | S | R | S |
| 3ETINI1 | *Citrobacter freundii* | R | R | R | R | R | R | R | R | R | R | R | R | R | S |
| 4EBC1 | *Kluyvera intermedia* | R | R | S | R | S | S | S | S | S | S | S | S | S | S |
| 4ETC4 | *Kluyvera intermedia* | R | R | S | R | S | S | S | S | S | S | S | S | S | S |
| 4EBINI1 | *Klebsiella pneumoniae* | R | R | R | R | R | R | R | R | R | R | S | S | R | S |
| 4EBINI2 | *Citrobacter amalonaticus* | R | R | R | I | R | I | R | R | R | R | S | R | R | S |

*AMP (Ampicillin), ASB (Ampicillin +sulbactam), PIT (Piperacillin + tazobactam), CFO (Cefoxitin), CAZ (Ceftazidime), CPM (Cefepime), CRO (Ceftriaxone), ETP (Ertapenem), IMI (Imipenem), MEM (Meropenem), AMI (Amikacin), GEN (Gentamicin), CIP (Ciprofloxacin), COL (Colistin) / Categories: S (Sensitive), I (Intermediate) and R (Resistant).The authors (2024).*

All isolates were resistant to ampicillin but sensitive to colistin. Within the group of beta-lactams with beta-lactamase inhibitors, enterobacteria showed a non-sensitivity of 79% (15/19) for ampicillin + sulbactam and 53% for piperacillin + tazobactam (10/19). Cefoxitin showed 68% (13/19), compared to 84% (16/19) ceftriaxone, 68% (13/19) ceftazidime, and 63% (12/19) cefepime. Regarding carbapenems, enterobacteria showed 42% (08/19) for ertapenem, imipenem and meropenem equally, compared to 26% (05/19) for amikacin and 11% (02/19) for gentamicin. Ciprofloxacin had a 74% (14/21) non-sensitivity profile.

Soriano-Moreno et al. conducted a study in hospital sewage reported that enterobacteria showed a resistance profile of 77% (25/32) for ampicillin + sulbactam, 100% for ampicillin, and 64% (20/32) for ciprofloxacin, corroborating the findings of the present study [52]. In contrast, higher resistance values compared to the findings of both WTPs regarding cefepime 94% (30/32), ceftazidime 94% (30/32), and gentamicin 40% (13/32) were noted. Nevertheless, the values for imipenem 27% (9/23), meropenem 13% (04/32), and piperacillin + tazobactam 13% (04/32) obtained from Soriano-Moreno et al. in Peru were significantly lower than the present study, suggesting that genera dispersion and the epidemiology of these bacteria in the study location can directly influence the profile of sensitivity to antimicrobials [51].

**3.3 Phenotypic detection of ESBL, AmpC, and carbapenemases**

Cephalosporin-resistant *Enterobacterales* were submitted to the disc approximation test for detecting ESBL and constitutive AmpC. Those non-sensitive to carbapenems were additionally submitted to phenotypic tests for carbapenemase, as shown in Table 3. The ESBL and AmpC tests were performed on the ninteen isolates, while the CLOXA, EDTA, AFB, and Hodge’s tests (HODGE) were performed on only eight isolates, which showed a profile of resistance to some carbapenem. Regarding the phenotypic test for ESBL, a positive result for 13 of the 19 isolates (68.4%) was obtained. For the six negatives for this test, five isolates were of the genus *Kluyvera* and one isolate of the specie *K. pneumoniae*, derived from the raw wastewater of STP B. Although this isolate was negative for ESBL, it showed positive phenotypic at the carbapenemase tests. The AmpC test was positive only for the species *C. freundii* isolated from the raw sewage of STP A.

**Table 3. Phenotypic tests and sensitivity to carbapenems in enterobacteria**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **ID** | **Species** | **ESBL** | **AmpC** | **AFB** | **EDTA** | **CLOXA** | **HODGE** | **ETP** | **IMI** | **MEM** |
| 1EBC1 | *Escherichia coli* | POS | NEG | NA | NA | NA | NA | S | S | S |
| 1EBINI2 | *Klebsiella pneumoniae* | POS | NEG | POS | NEG | NEG | POS | R | R | R |
| 1EBINI3 | *Klebsiella pneumoniae* | POS | NEG | POS | NEG | NEG | POS | R | R | R |
| 2EBINI3 | *Citrobacter farmeri* | POS | NEG | POS | NEG | NEG | POS | R | R | R |
| 2EBINI4 | *Klebsiella pneumoniae* | POS | NEG | POS | NEG | NEG | POS | R | R | R |
| 2ETC1 | *Escherichia coli* | POS | NEG | NA | NA | NA | NA | S | S | S |
| 2ETC4 | *Kluyvera cryocrescens* | NEG | NEG | NA | NA | NA | NA | S | S | S |
| 2ETC5 | *Kluyvera cryocrescens* | NEG | NEG | NA | NA | NA | NA | S | S | S |
| 2ETINI1 | *Kluyvera cryocrescens* | NEG | NEG | NA | NA | NA | NA | S | S | S |
| 3EBC2 | *Enterobacter asburiae* | POS | NEG | NA | NA | NA | NA | S | S | S |
| 3EBC3 | *Citrobacter freundii* | POS | POS | NA | NA | NA | NA | S | S | S |
| 3EBC4 | *Escherichia coli* | POS | NEG | NA | NA | NA | NA | S | S | S |
| 3EBINI1 | *Klebsiella pneumoniae* | POS | NEG | POS | NEG | NEG | POS | R | R | R |
| 3ETC2 | *Citrobacter freundii* | POS | NEG | NA | NA | NA | NA | S | S | S |
| 3ETINI1 | *Citrobacter freundii* | POS | NEG | NEG | POS | NEG | NEG | R | R | R |
| 4EBC1 | *Kluyvera intermedia* | NEG | NEG | NA | NA | NA | NA | S | S | S |
| 4ETC4 | *Kluyvera intermedia* | NEG | NEG | NA | NA | NA | NA | S | S | S |
| 4EBINI1 | *Klebsiella pneumoniae* | NEG | NEG | POS | NEG | NEG | POS | R | R | R |
| 4EBINI2 | *Citrobacter amalonaticus* | POS | NEG | POS | NEG | NEG | POS | R | R | R |

*ESBL (Extended Spectrum Beta-lactamases), AmpC (Ambler's Class C Ampicillinase), AFB (Phenylboronic Acid), EDTA (Ethylenediaminetetraacetic Acid), CLOXA (Cloxacillin), ETP (Ertapenem), IMI (Imipenem), MEM (Meropenem) / Categories: POS (Positive), NEG (Negative), S (Sensitive), R (Resistant) and NA (Not applicable). The authors (2024).*

For one of the carbapenems tested (imipenem, meropenem, and ertapenem) phenotypic tests for carbapenemase for isolates of enterobacteria non-sensitive were performed. Initially, Hodge’s test was performed for nonspecific screening of this type of resistance [25]. Seven of the eight isolates (87.5%) were positive for the test, except for one *C. freundii* isolate derived from a sample of treated sewage from the STP B. The test was performed with the three carbapenems to increase its sensitivity [16]. Phenotypic tests are an essential screening tool for such resistance mechanisms, given that most microbiology laboratories in Brazil still lack the molecular biology methodology for detecting the genera that produce the enzymes in question [33]. The results were the same for the three tested antimicrobials.

The test with CLOXA was negative for all samples, indicating no evidence of AmpC-associated porin losses. The EDTA test was positive for only one *C. freundii* isolate, derived from a sample of treated sewage from STP B, indicating that the bacterium carries a metallo-beta-lactamase, requiring a molecular test to designate which genus produces it [25].

The AFB was positive in seven isolates (87.5%). The *C. freundii* isolate was the only negative for the AFB test and the only positive for the EDTA test. A positive test for AFB indicates the presence of serine-carbapenemase, with KPC comprising the most known and frequently detected type [25]. Santos et al. also conducted disk-approximation tests for ESBL, Hodge’s test for detecting carbapenemases, and enzymatic blockade for metallo-beta-lactamases in enterobacteria, similar to the present study. The enterobacteria studied were derived from a hospital located in Foz do Iguaçu, Paraná. Previously published literature, the bacterium *K. pneumoniae* was the most frequently associated with the phenotypic test with AFB, indicating the probable presence of KPC-type carbapenemases [53]. Other methodologies are also employed to detect metallo-beta-lactamases in enterobacteria, such as enzymatic blockade with EDTA and other reagents (i.e., 2-MPA) [25, 54, 55].

All carbapenem-resistant isolates were detected in STP B samples, which receive hospital sewage, significantly contributing to the isolation of *K. pneumoniae* and *Citrobacter sp.* with some positive phenotypic tests for carbapenemases [3, 31, 34, 35].

**3.4 Research on genes for resistance to beta-lactam antimicrobials**

Molecular tests were performed to research genera blaKPC, blaIMP, blaVIM, blaBKC, blaOXA23, blaOXA48, and blaCTX-M in eight isolates, which showed positive results in the phenotypic test. Except for blaKPC, all other genus sequences were not detected. Six tested isolates (75%) evidenced blaKPC: four isolates of *K. pneumoniae* (1EBINI2, 1EBINI3, 2EBINI4, and 3EBINI1), one isolate of *C. farmeri* (2EBINI4) and one of *C. amalonaticus* (4EBINI2), all originating from the non-treated sewage of the STP B, in all collections, which indicates that the blaKPC genus is constantly found in the non-treated sewage of the STP, also suggesting its presence in the hospital center.

Considering the results obtained for the phenotypic tests, all isolates with detected blaKPC were also positive for AFB and Hodge’s tests, indicating that the tests in question were 100% sensitive and could be used to screen this mechanism. This result corroborates previous studies by our group, which indicated using these phenotypic tests as a valuable presumptive guideline for assessing the presence of carbapenemases in enterobacteria [33].

Two isolates, *C. freundii* (3ETEINI1) and *K. pneumoniae* (4EBINI1) did not show the searched sequences, which may be explained by the presence of other genes different from those investigated in this study. In the 3ETEINI1 sample, the EDTA test was positive, and the blaVIM and blaIMP sequences (metallo-beta-lactamases) were not detected. Some other genes for metallo-beta-lactamases may be present, requiring additional tests with other sequences.

Nonetheless, the 4EBINI1 isolate tested positive for AFB and Hodge, suggesting that some other gene from the serine group may likely to be present even if there is no detection for blaKPC and blaBKC. Other carbapenemases have been reported, such as Guiana extended-spectrum (GES), Non-metallo carbapenemase-A (NMC), Imipenemase (IMI), and *Serratia marcescens* enzyme SME [56].

Genera blaKPC (57.5%), blaVIM (30.2%), blaGES (17%), blaNDM (15%), and blaSPM (2.4%) were detected in carbapenem-resistant GNB isolated from a hospital located at the Brazilian state of Pernambuco. Although the isolates were derived from clinical samples, the frequency of blaKPC was significant, as in the present study. However, regarding blaVIM with 30.2% frequency in the mentioned study, such a genus was not detected in our research. The blaGES, blaNDM, and blaSPM genera were not researched [57]. In a study conducted by Batista, 81% of genera blaKPC isolates from *K. pneumoniae* were obtained [55]. Phenotypic tests were run before the molecular tests and obtained positivity for AFB in 165 isolates. Soon afterward, the blaKPC genus was detected in 162 isolates, showing a sensitivity of 100%. The specificity of the phenotypic test concerning molecular tests does not reach this number, but it reaches 98% in the cited study and 85% in our research [25].

According to Abrantes and Nogueira, while not being the most modern or definitive methodology, such as molecular analyses for the final diagnosis of carbapenemases, phenotypic methods contribute in a very positive and economical way in the laboratory environment, unquestionably assisting in the perception of bacterial resistance in researched strains [33]. The results obtained in this study show the predominant KPC type in Brazil as previously reported [25, 55, 57-60]. Such information is essential to relate to epidemiological studies, confirming the presence of this type of resistance and the risk to public health. In the environmental context, bacteria carrying resistant genes risk transferring them to other bacteria, leading to an imbalanced environment by modifying ecosystems, such as affecting aquatic biota, and limited resistance through consumption or resistance of aquaculture animals [47].

The results obtained emphasize the dissemination of potentially pathogenic and antimicrobial-resistant microorganisms in the environment from STP’s final effluents, regardless of the type of system adopted [61]. Furthermore, abiotic variables, such as temperature, pH, and electrical conductivity, are capable of directly influence the microbial composition of effluents, modulating the development of specific bacterial groups with distinct physiological and biochemical profiles [62]. These types of effluent should undergo additional treatment and disinfection to reduce this release significantly [60]. Chlorine disinfection is already applied in STP B, effectively reducing the microbial load of the sewage, including bacteria resistant to the main antimicrobials, as the researched resistance genes were not detected in the treated sewage of this station [63, 64].

4. Conclusion

The two WTPs analyzed detected *Enterobacterales* that are resistant and multi-resistant to several antimicrobials. A high profile of non-sensitivity (resistant + intermediate) to most antimicrobials, especially third-generation cephalosporins, quinolones, and carbapenems were reported. The phenotypic tests were effective in screening the mechanisms of resistance to carbapenems. They were compatible with the molecular tests, showing the blaKPC gene as the only one detected among those surveyed. It reflects the epidemiological predominance of this genus in enterobacteria in Brazil.

Finally, enterobacteria resistant to carbapenems were isolated in treated and non-treated sewage only from STP B, confirming the hospital origin of these microorganisms. The concern related to environmental contamination and the spread of bacterial resistance to antimicrobials in aquatic reservoirs must be addressed.

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References

1. Amaro, A.; Silva, A.; Motafa, A.; Costa, C.; Morza, A.; Oliveira, I. The importance of good solid waste management: Case report. Facit Business and Technology Journal. 2018; 8, 45-52.

2. Mendonça, M. H. M.; Rosen, O, S. A. M.; Waterfall, T. R. L.; Silva, A. F. S.; Jacome, P. R. L. A.; Jácome Júnior, A. T. Bacteriological analysis of drinking water sold by tanker trucks. Environment & Water Magazine. 2017; 12, 468-475.

3. Zhang, Y.; Chen, L.; Sun, R.; Dai, T.; Tian, J.; Liu, R.; Wen, D. Effect of wastewater discharge on the bacterial and archaeal community of marine sediments in an industrial area in China. FEMS Microbiology Ecology. 2014; 88(2), 320-332.

4. ITB - Trata Brazil Institute. Electronic portal, 2018. Available online: <http://www.tratabrasil.org.br/saneamento-no-brasil>. (Accessed in Sep. of 2021). (In Portuguese).

5. Brazil. Ministry of Health. National Health Foundation. Sanitation Manual. 4th. ed. – Brasilia, 2015. (In Portuguese)

6. Ribeiro, E. A.; De Oliveira, R. A.; Da Gama Melo, J. D.; Da Silva, G. T. P.; Carneiro, J. L. S.; Da Silva, I. K. L. Phenotypic detection of Gram-negative bacteria producing carbapenemases in hospital effluent in the Amazon in the state of Pará, Brazil. Journal of Environmental Sciences and Health. 2020; 47, 7939. (In Portuguese).

7. Adeolu, M.; Alnajar, S.; Naushad, S.; Gupta R. S. Genome-based phylogeny and taxonomy of the ‘Enterobacteriales’: Proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. Nov. International Journal of Systematic and Evolutionary Microbiology. 2016; 66, 5575–5599.

8. Murray, PR; Baron, E. J.; Jorgensen, J.H.; Pfaller, M. A.; Yolken, HR Manual of Clinical Microbiology. 8th ed. ASM Press: Washington, 2017; 2113p.

9. Koneman, EW; Allen, SD; Janda, W. M.; Schreckenberger Pc, Winn, J.R.W.C. Microbiological Diagnosis - Text and Color Atlas. 7th ed. Medsi: Rio de Janeiro, 2018; 1860p.

10.Tang, K. W. K., Millar, B. C., Moore, J. E. Antimicrobial resistance (AMR). British journal of biomedical science. 2023; 80, 11387.

11. Alves, F. C. B. Mechanisms of action of the antibacterial activity of nisin and in combinations with traditional antimicrobials against methicillin-resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa. Doctoral Dissertation (PhD in General and Applied Biology – IBB). UNESP. Sao Paulo, 2018. (In Portuguese).

12. Girlich, D.; Grosperrin, V.; Naas, T.; Dortet, L. CHROMagar™ ESBL/mSuperCARBA bi-plate medium for detection of ESBL- and carbapenemase-producing Enterobacteriaceae. Diagnostic Microbiology and Infectious Disease. 2019; 95, 107-112.

13. APHA, AWWA, WEF. Standard Methods for the examination of water and wastewater. Washington, DC. American Public Health Association, America Water Works Association, Water Poll. Control Federation, 23rd ed., 2017.

14. Hornsey, M.; Phee, L.; Woodford, N.; Turton, J.; MEUnier, D.; Thomas, C.; Wareham, D.W. Evaluation of three selective chromogenic media, CHROMagar ESBL, CHROMagar CTX-M, and CHROMagar KPC, for the detection of Klebsiella pneumoniae producing OXA-48 carbapenemase. Journal of Clinical Pathology. 2013; 66, 348–350.

15. Ceballos, B.S.O.; Diniz, C.R. Sanitary and environmental microbiology techniques. EDUEBP: Campina Grande, 2017; 324p.

16. CLSI. Institute of Clinical and Laboratory Standards. Performance standards for antimicrobial susceptibility testing. CLSI/NCCLS M100–29th ed. Wayne, PA, USA, 2019. (In Portuguese).

17. Costa, G. A.; Hofer, E. Isolation and identification of Enterobacteriaceae. Monograph, Inst. Inst. Oswaldo Cruz, Rio de Janeiro, 1972.

18. Cunha, M.A.; Silva, M.R. Methods for detecting indicator microorganisms. Health & Environment in Review. 2006; 1, 9-13. (In Portuguese).

19. BrCAST - Brazilian Committee on Antimicrobial Susceptibility Testing, 2019. Available online: http://brcast.org.br. (Accessed in Sep. of 2021).

20. Jarlier, V.; Nicolas, M.H.; Fournier, G.; Philippon, A. Extended broad-spectrum β-lactamases providing transferable resistance to newly arrived β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Infectious Disease Reviews. 1988; 10, 867-878.

21. Lee, K.; Chong, Y.; Shin, H.B.; Kim, Y.A.; Yong, D.; Yum, J.H. Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase producing strains of Pseudomonas and Acinetobacter species. Clinical Microbiology and Infection. 2001; 7, 88-91.

22. CLSI. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 25th Informational Supplement. CLSI document M100- S25. Wayne, PA, USA, 2015.

23. Pasteran, F.; Mendez, T.; Guerriero, L.; Rapoport, M.; Corso, A. Sensitive Screening Tests for Suspected Class A Carbapenemase Production in Species of Enterobacteriaceae. Journal of Clinical Microbiology. 2009; 47, 1631–1639.

24. Vali, P.; Shahcheraghi, F.; Seyfipour, M.; Zamani, M. A.; Allahyar, M. R.; Feizabadi, M. M. Phenotypic and genetic characterization of carbapenemase and ESBLs producing Gram-negative bacteria (GNB) isolated from patients with cystic fibrosis (CF) in Tehran hospitals. Journal of clinical and diagnostic research. 2014; 8, 26.

25. ANVISA - Agência Nacional de Vigilância Sanitária. Nota técnica nº 01/2013 – Medidas de prevenção e controle de infecções por enterobactérias multirresistentes. Brasília, 2013. (In Portuguese).

26. Garbati, M.A.; Al Godhair, A.I. The growing resistance of Klebsiella pneumoniae; the need to expand our antibiogram: case report and review of the literature. African Journal of Infectious Diseases. 2013; 7, 8–10.

27. Hasman, H.; Mevius, D.; Veldman, K.; Olesen, I.; Aarestrup, F.M. β-lactamases among extended-spectrum β-lactamase (ESBL)-resistant Salmonella from poultry, poultry products and human patients in The Netherlands. Journal of Antimicrobial Chemotherapy. 2005; 56, 115-121.

28. Lima, P.M. Caracterização genética de isolados clínicos e ambientais de Klebsiella pneumoniae. Master's Thesis (Master's Degree in Parasite Biology) – Instituto Oswaldo Cruz, 2011. (In Portuguese).

29. Freitas, F. S. Caracterização genética e genômica de Helicobacter pylori. Master's Thesis (master’s degree in computational and systems Biology) Fundação Oswaldo Cruz, Rio de Janeiro, 2015. (In Portuguese).

30. Ygiti, H.; Queenan, A.M.; Anderson, G.J.; Domenech-Sanchez, A.; Biddle, J.W.; Steward, C.D.; Alberti, S.; Bush, K.; Tenover, F.C. Novel Carbapenem-Hydrolizing β- Lactamase, KPC-1, from a Carbapenem-Resistant Strain of Klebsisella pneumoniae. Antimicrobial Agents and Chemotherapy. 2001; 45, 1151-1161.

31. Nicoletti, A.G.; Marcondes, M.F.; Martins, W.M.; Almeida, L.G.; Nicolas, M.F.; Vasconcelos, A.T.; Oliveira, V.; Gales, A. C. Characterization of BKC-1 class A carbapenemase from Klebsiella pneumoniae clinical isolates in Brazil. Antimicrobial Agents and Chemotherapy. 2015; 59, 5159-5164.

32. Mulvey, M.R.; Soule, G.; Boyd, D.; Demczuk, W.; Ahmed, R. Multi-provincial Salmonella Typhimurium case-control study group. Characterization of the first extended-spectrum beta-lactamase-producing Salmonella isolate identified in Canada. Journal of Clinical Microbiology. 2003; 41, 460-462.

33. Abrantes, J.A.; Nogueira, J.M.R. Utilização de testes fenotípicos para a pesquisa de carbapenemases em enterobactérias: uma ferramenta para orientação clínica. Revista Brasileira de Análises Clínicas. 2017; 49, 240-244. (In Portuguese).

34. Picão, R.C.; Cardoso, J.P.; Campana, E.H.; Nicoletti, A.G.; Petrolini, F.V.; Assis, D.M.; Juliano, L.; Gales, A.C. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing Aeromonas spp. and Enterobacteriaceae in sewage. Diagnostic Microbiology and Infectious Disease. 2013; 76, 80-5.

35. Zagui, G. S. Avaliação da multirresistência a antibióticos e produção de ESBL e carbapenemases em bacilos Gram-negativos de efluente hospitalar e urbano. Master's Thesis (master’s in public health nursing) - Escola de Enfermagem de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2019.

36. Lopes, T.R.; Costa Jr. I.L.; Periotto, F.; Pletsch, A.L. Antibiotic resistance in E. coli isolated in effluent from a wastewater treatment plant and sediments in receiver body. International Journal of River Basin Management. 2016; 14, 441-445.

37. Zhang, S.; Han, B.; Gu, J.; Wang, C.; Wang, P.; Ma, Y.; Cao, J.; He, Z. Fate of antibiotic-resistant cultivable heterotrophic bacteria and antibiotic resistance genera in wastewater treatment processes. Chemosphere. 2015; 135, 138-145.

38. Facciolà, A.; Virga, A.; Gioffrè, M.E.; Laganà, P. Evaluation of Antibiotic Resistance in Bacterial Strains Isolated from Sewage of Slaughterhouses Located in Sicily (Italy). International journal of environmental research and public health. 2021; 18, 9611.

39. Puljko, A.; Milakovic, M.; Krizanovic, S.; Kosić-Vukšić, J.; Babic, I.; Petric, I.; Maravić, A.; JELIć, M.; Udiković-Kolić, N. Prevalence of enteric opportunistic pathogens and extended-spectrum cephalosporin and carbapenem coliforms and genera in wastewater from municipal wastewater treatment plants in Croatia. Hazardous Materials Journal. 2022; 427, 128155.

40. Martins, J.C.L. Detection and molecular characterization of antibiotic-resistant enterobacteriaceae isolates in ready-to-eat fresh products. Master's Thesis (master’s in food safety) Faculty of Pharmacy, University of Coimbra, 2021. (In Portuguese).

41. Gonzalez-Romero, A.C.; Guamán-Chabla, M.G.; Cordovez-Martinez, M. Del C.; Martinez-Duran, E.E. Antimicrobial susceptibility profiles of bacteria isolated from agricultural crops in the Chambo river basin. Profiles. 2022; 1, 39-48.

42. Cabeço, A.L.B.; Colombo, T. Bacteria causing urinary tract infections and their antimicrobial resistance profile. Journal of the Institute of Health Sciences. 2019; 37, 113-8.

43. Marson, P.G.; Rodrigues Nepomuceno, V.; Eugene, F.; Neves, T. N.; Silva, M.; Soares, M.; Mundim, A. P. Association between oral biofilm and tracheal aspirate in patients with ventilator-associated pneumonia. Cereus Magazine. 2020; 12, 272-288.

44. Li, Y.; Luo, L.; Xiao, Z.; Wang, G.; Li, C.; Zhang, Z.; Zhou, Y.; Zhang, L. Characterization of a Carbapenem-Resistant Kluyvera cryocrescens Isolate Carrying Blandm- 1 from Hospital Sewage. Antibiotics. 2019; 8, 149.

45. Wang, L.; Jing, Y.; Lai, K.; An, J.; Yang, J. A case of biliary tract infection caused by KPC-2 producing Kluyvera ascorbata. Case Reports in Infectious Diseases. 2018; 2018.

46. Hernandez-Garcia, M.; Leon-Sampedro, R.; Perez-Viso, B.; Morosini, M. I.; Lopez-Fresneña, N.; Says-Agero, C.; Coque, T. M.; Ruiz-Garbajosa, P.; Cantón, R. First report of an OXA-48- and CTX-M-213-producing Kluyvera species clone recovered from patients admitted to a university hospital in Madrid, Spain. Antimicrobial Agents and Chemotherapy. 2018; 62, 01238-18.

47. Silva, T.S.M. Microbiological quality of the Carioca River-RJ: isolation and identification of bacteria from the thermotolerant coliform group and their susceptibility to antimicrobials. Master's Thesis (Master's Degree in Public Health and the Environment). Oswaldo Cruz Foundation, 2021. (In Portuguese).

48. Salimiyan Rizi, K.; Ghazvini, K.; Farsiani, H. Clinical and pathogenetic overview of Enterobacter infections. Assessments in Clinical Medicine. 2020; 6, 146-154.

49. Lima, M.M. From S.; Fernandes, D.G.G., De Oliveira, E.A.; Pinheiro, M. P.; FERREIRA, D. G.; Zahner, V.; CHAGAS, T.P.G.; From Mendonca-Souza, C.R.V. Detection of carbapenemase-producing Enterobacterales in colonized patients treated at a University Hospital. Electronic Journal Health Collection. 2021; 13, 10. (In Portuguese).

50. Wu, W.; Wei, L.; FEng, Y.; Xie, Y.; Zong, Z. Precision Species Identification by Whole-Genome Sequencing of Enterobacter Bloodstream Infection, China. Emerging Infectious Diseases. 2021; 27, 161–169.

51. Baker, C.; Gardner, C. Diagnosis Suspected by Mechanism of Injury: Soft Tissue Infection Due to Aeromonas hydrophila and Enterobacter asburiae Following Human Wastewater Exposure. Journal of Urgent Care Medicine. 2021; 31-33.

52. Soriano-Moreno, D.R.; Yareta, J.; Rojas-Cosi, A.F.; Fajardo-Loyola, A.; Leon-Luna, D.; Castillo-Quezada, I.; Marcos-Carbajal, P. Hospital effluents as a reservoir of beta-lactamase and carbapenemase-producing enterobacteria. Peruvian Journal of Experimental Medicine and Public Health. 2021; 38, 302-307.

53. Santos, F.F.S.; Son, J. D.; MACHADO, C. T.; VASCONCELOS, J. F.; FEITOSA, F. R. S. The development of basic sanitation in Brazil and the consequences for public health. Brazilian Journal of the Environment. 2018; 4, 241-251. (In Portuguese).

54. From Birth, A. C.; Reichardt, V. T.; Vasco, J. F.; RODRIGUES, L. S. Phenotypic tests for detection and differentiation of carbapenemases in Enterobacterales isolated from blood cultures of oncology patients. Notebooks of the School of Health. 2019; 19, 40-49. (In Portuguese).

55. Baptist, A.C.C.A. Klebsiella Pneumoniae: Phenotypic and Molecular Analysis of KPC and NDM Resistance Mechanisms in a Hospital in Foz do Iguaçu, PR. Master's Thesis (Master of Science). Federal University for Latin American Integration. Foz do Iguaçu, 2020. (In Portuguese).

56. Da Silva, R.P.P.; MANÇANO, S.M.C.N.; Picão, R. C. Chronology of the global emergence of carbapenemases in Gram-negative bacilli. Saber Digital Magazine. 2017; 10, 43-61. (In Portuguese).

57. Soares, C.R.P.; DA SILVA, F.R.F.; JUNIOR, J.B.O.; From Araujo, P. S. R.; Firmo, E. F. Molecular epidemiology of multidrug-resistant Gram-negative bacilli producing carbapenemases isolated from different sites of infection. Research, Society and Development. 2021; 10, e30210918070-e30210918070. (In Portuguese).

58. Rodrigues, A. C. S., Chang, M. R., Santos, I. C. D. O., & Carvalho-Assef, A. P. D. A. (2022). Molecular Epidemiology of bla KPC-Encoding Klebsiella pneumoniae Isolated from Public Hospitals in Midwest of Brazil. *Microbial Drug Resistance*, *28*(1), 1-6.

59. De Souza, A.B.A.; RAMALHO, F. L.; Camargo, B. Prevalence of nosocomial infections caused by carbapenemase-producing Klebsiella pneumoniae (KPC) in hospitalized individuals. Brazilian Journal of Health Review. 2020; 3, 1915-1932. (In Portuguese).

60. Conceição-Neto, O. C., da Costa, B. S., Pontes, L. D. S., Silveira, M. C., Justo-da-Silva, L. H., de Oliveira Santos, I. C., ... & Carvalho-Assef, A. P. (2022). Polymyxin resistance in clinical isolates of K. pneumoniae in Brazil: Update on molecular mechanisms, clonal dissemination and relationship with KPC-producing strains. *Frontiers in Cellular and Infection Microbiology*, *12*, 898125.

61. Araújo S, Sousa M, Tacão M, Baraúna RA, Silva A, Ramos R, Alves A, Manaia CM, Henriques I. Carbapenem-resistant bacteria over a wastewater treatment process: Carbapenem-resistant Enterobacteriaceae in untreated wastewater and intrinsically-resistant bacteria in final effluent. Science of the Total Environment. 2021 Aug 15;782:146892.

62. Manaia CM, Rocha J, Scaccia N, Marano R, Radu E, Biancullo F, Cerqueira F, Fortunato G, Iakovides I, Zammit I, Kampouris I, Vaz-Moreira I, Nunes OC. Antibiotic resistance in wastewater treatment plants: Tackling the black box*. Environment International*, 2018; 115:312–324.

63. Li, Y., Yang, M., Zhang, X., Jiang, J., Liu, J., Yau, C. F., Graham N. J. D., Li, X. Two-step chlorination: A new approach for disinfection of a primary drought effluent. Water Research. 2017; 108, 339-347.

64. Ocampo-Rodríguez, D. B., Vázquez-Rodríguez, G. A., Martínez-Hernández, S., Iturbe- Acosta, U., Coronel-Olivares, C. Water disinfection: a review of conventional and advanced treatments with chlorine and peracetic acid. Water engineering. 2022; 26, 185-204.