*Original Research Article*

Evaluating Bacterial Resistance In Sewage Treatment Plants: Detection Of KPC-Producing Enterobacteria In Wastewater, Brazil

.

ABSTRACT

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| **Background and Aims:** Pathogenic microorganisms can be found in sewage and may cause several infections and consequent deleterious health effects, especially in humans. Bacteria belonging to the Enterobacterales order can trigger several diseases in humans and animals and disseminate resistance genera in this environment.This study aims to identify Enterobacterales bacteria isolates from Sewage Treatment Plants (STP) in Brazil.  **Methodology:** Biochemical screening assays and antimicrobial susceptibility tests (AST) previously established by international guidelines were applied for bacteria identification. A chromogenic medium infused with third-generation cephalosporin was employed, followed by antimicrobial susceptibility tests (AST), phenotypic assays for carbapenemase detection, and the identification of genes related to bacterial resistance to third and fourth generation cephalosporins and carbapenems. Samples were processed in the Microbiology Laboratory (Labmicro) of the Department of Biological Sciences at the Sérgio Arouca National School of Public Health – FIOCRUZ  **Results:** Eight species were identified in the 19 Enterobacterales isolates. 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. These findings indicate the presence of the blaKPC gene, reflecting the predominance of this enterobacteria in Brazil. Phenotypic tests effectively screened carbapenem resistance mechanisms, displaying high concordance with molecular tests.  **Conclusion:** A high profile of non-sensitivity to most antimicrobials tested, especially third-generation cephalosporins, quinolones, and carbapenems were reported. It reflects the epidemiological predominance of this genus in enterobacteria in Brazil. 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 the environment, mainly because it carries 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 have proposed new families, and currently, the order comprises seven families: *Enterobacteriaceae*, *Erwiniaceae*, *Morganellaceae*, *Yersiniaceae*, *Pectobacteriaceae*, *Hafniaceae*, and *Budviciaceae* [7]. They are 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 the 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. They were named A and B. Laboratory-borne effluents were treated at STP A and effluents from hospital origin at STP B. The STP B was 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**

A chromogenic culture medium containing antimicrobial cefotaxime of the third-generation 86 cephalosporin class (2 mg/L), CHROMagar ESBL (Plast Labor®), was employed. 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]. Identification was performed with an automated method in VITEK 2® (BioMérieux), an automatic photometric reading device.

**2.3 Antimicrobial Sensitivity Test**

Sensitivity of the isolated microorganisms was evaluated using the agar diffusion methodology (disk diffusion) in the Müeller-Hinton agar medium, and antibiotic disks (Oxoid®) suitable for *Enterobacterales* [16,19]. Several antimicrobials were used from compendiums (Guidelines) accepted in the country to perform the Antimicrobial Sensitivity Test (AST) such as the Clinical and Laboratory Standards Institute (CLSI) and the Brazilian Antimicrobial Sensitivity Testing Committee (BrCAST) [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 the 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. In previous 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, 57-59]. 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. 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 [60, 61].

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.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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