

Wastewater surveillance for identification of *Enterobacteriales* priority pathogens list from the World Health Organization

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Highlights:

- We detected priority pathogens resistant to third generation cephalosporins and carbapenems in the sewage.
- The most prevalent genera in wastewater from both WWTPs were *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella*.
- Combining culturing and sequencing approaches improved our ability to identify priority pathogens.

Keywords: bacteria resistant; *Enterobacteriales*; wastewater surveillance; World Organization Health.

INTRODUCTION

The development of antibiotics represents one of the most significant advancements in modern medicine, saving countless lives across human and animal populations. However, the widespread overuse of these medications has led to the emergence of antibiotic-resistant bacteria, complicating the treatment of infections. In 2024, the World Health Organization (WHO) updated its Bacterial Priority Pathogens List, identifying all *Enterobacteriaceae* resistant to third-generation cephalosporins and carbapenems as a critical priority group (WHO, 2024).

Among antibiotics, beta-lactams are the most commonly prescribed class in many countries, including Brazil (ECDC, 2022; WHO, 2017). The rising resistance of bacteria to this class poses a significant threat to patient care, public health, and animal health. Carbapenems, a subgroup of β -lactam antibiotics with broad-spectrum activity, are often reserved for severe infections caused by extended-spectrum beta-lactamase (ESBL) producers.

Addressing antimicrobial resistance (AMR) in human populations is crucial for healthcare systems to prioritize effective public health actions. However, current clinical surveillance methods are resource-intensive, requiring numerous patient samples and substantial financial support. In contrast, our comprehensive study on AMR in wastewater reveals an alarming yet underexplored avenue for monitoring resistance trends. By examining pathogens in wastewater, we can gain critical insights into the broader epidemiological landscape of AMR, paving the way for more efficient and proactive public health strategies.

METHODOLOGY

We monitored raw sewage samples from two wastewater treatment plants, Arrudas and Onça, in Belo Horizonte between 2022 and 2024. We diluted and filtered the samples using membranes with a 0.45 µm porosity and a 47 mm diameter. Afterwards, we placed the membranes on ESBL and KPC ChromoSelect Agar plates, both with and without supplements, to select resistant bacteria. In duplicate, we incubated the plates at 37°C for 18-24 hours.

We used one membrane to isolate microorganisms and identify them with MALDI-ToF. From the other membrane, we extracted DNA using the FastKitDNA (MPBiologics) protocol. To sequence the 16S rRNA gene, we followed Oxford Nanopore's Barcode kit (SQK-16S024) protocol. For data analysis, we used Oxford Nanopore's EPI2ME platform and its 16S-BLAST tool, which classified the barcode sequences and sorted the reads into their corresponding taxonomic folders.

RESULTS AND CONCLUSIONS

The concentration of extended-spectrum beta-lactamase (ESBL)-resistant bacteria in the wastewater was measured at $4.80 \pm 0.336 \log_{10}$ CFU/mL, while carbapenemase-producing *Klebsiella pneumoniae* (KPC)-resistant bacteria exhibited a concentration of $3.56 \pm 0.690 \log_{10}$ CFU/mL. The relative abundance of ESBL-resistant bacteria accounted for 76.9%, and KPC-resistant bacteria represented 56.9% of the total bacterial population in the sewage (Figure 01).



Figure 1 Comparison of resistant and non-resistant bacteria in two WWTP during 2022 to 2024

The analysis of the 16S rRNA gene from culture plates for both ESBL- and KPC-resistant bacteria revealed a diverse community of the *Enterobacteriales* order in sewage, as shown in Figure 2. Out of the 36 previously identified genera within the Enterobacteriaceae family, eight exhibited an abundance greater than 1%. These included *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kasokonia*, *Raoultella*, *Serratia*, and *Shigella*. According to the World Health Organization (WHO, 2024), *Escherichia coli*, *Klebsiella pneumoniae*, and *Shigella* spp. are highly transmissible pathogens, with

documented cases of molecular or genetic resistance profile transmission. The identification of these genera was initially determined through 16S rRNA sequencing and further confirmed through MALDI-ToF analysis.

Among the species isolated from ESBL-resistant cultures, we identified *E. coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Raoultella ornithinolytica*, and *Enterobacter kobei*. Notably, *Klebsiella pneumoniae* was the most prevalent species isolated from KPC media. These findings align with previous studies (HENDRIKSEN *et al.*, 2019; TIWARI *et al.*, 2022), reinforcing the notion that integrating complementary methodologies, such as culturing and sequencing, significantly improves the capacity of public health systems to identify circulating priority pathogens in human populations. Furthermore, *Citrobacter*, *Enterobacter*, and *Serratia* exhibit moderate levels of transmissibility. Understanding these bacteria's transmission dynamics and resistance profiles is crucial in assessing the associated public health risks.

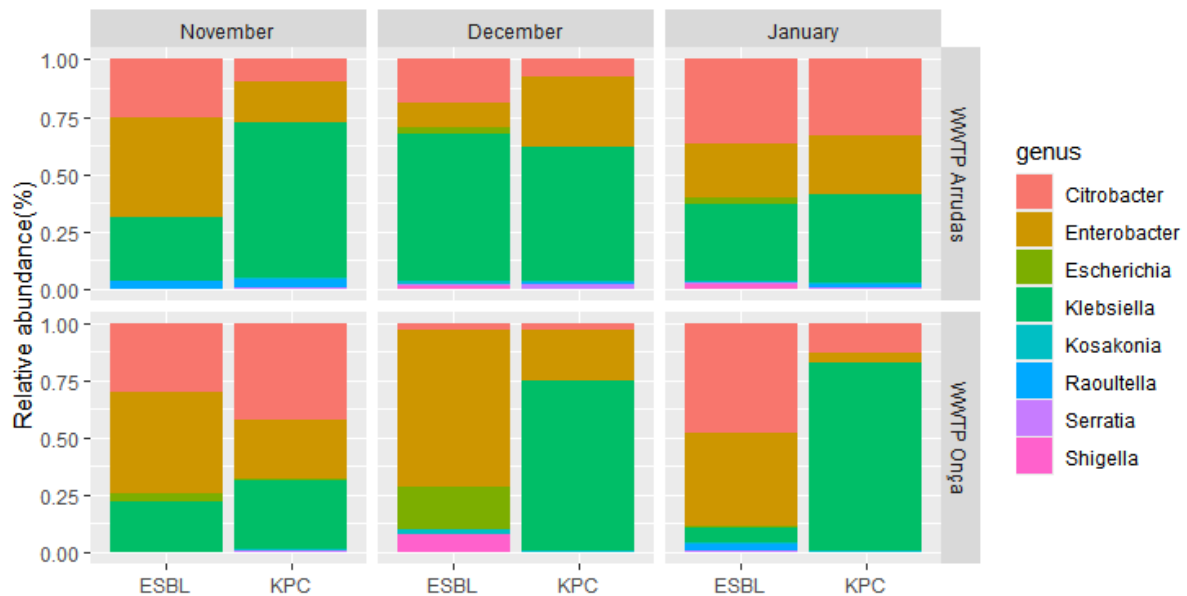


Figure 2 Relative abundance of members of the *Enterobacteriales* order resistant to ESBL and KPC, present in untreated sewage from Belo Horizonte WWTPs

In conclusion, while our study's methodologies—culturing, 16S rRNA sequencing, and MALDI-ToF—provide critical insights into resistant bacteria in wastewater, simply monitoring these indicators without linking them to prevention or treatment strategies limits their practical impact. Identifying priority pathogens like *E. coli*, *Klebsiella pneumoniae*, and *Shigella* spp. is essential, but integrating these findings with rapid detection systems and intervention techniques is critical to mitigating their spread. This work underscores the importance of understanding resistance profiles in wastewater. Still, to truly protect public health, these surveillance efforts must be paired with actionable solutions that reduce the prevalence of these pathogens in the environment and prevent their transmission.

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