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ESCOLA DE MEDICINA E SAÚDE PÚBLICA

CURSO DE GRADUAÇÃO EM BIOMEDICINA

ANA BEATRIZ PURIFICAÇÃO DE SOUZA

RESISTÊNCIA AOS ANTIMICROBIANOS ENTRE ISOLADOS DE *ESCHERICHIA COLI*  
EM AMOSTRAS HÍDRICAS DO RIO DO COBRE, SALVADOR, BRAZIL

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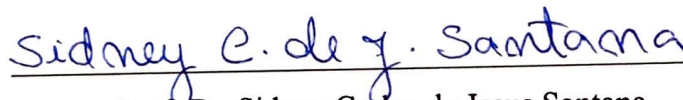
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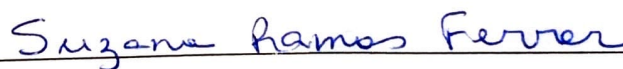
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## SUMÁRIO

1. ARTIGO .....	5
2. REGRAS DE SUBMISSÃO .....	19

**Title:** Antimicrobial-resistant in *Escherichia coli* isolates from water samples of Cobre River, Salvador, Brazil (16 pages)

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## Abstract

Poor sanitation has a strong influence on fecal contamination in urban surface waters and contributes to increase and propagate antibiotic resistance in the ecosystem. This study focused on describing the occurrence of antibiotic-resistant bacteria and genes in a river system in an urban area in Brazil. Water samples were collected from 7 different sampling points of the Cobre River and analyzed for antibiotic-resistant bacteria. The isolates were identified using MALDI-TOF<sup>®</sup> and the antibiogram profile by VITEK<sup>®</sup>. The presence of antibiotic resistance genes (ARGs) was studied using conventional polymerase chain reaction (PCR). It was observed ARGs and antibiotic-resistance bacteria in all study sites. In 126 *Escherichia coli* isolates from environmental samples revealed that 59% of the isolates were resistant to ciprofloxacin, 44% to amoxicillin/clavulanic acid and 42% to aztreonam. For the cephalosporin group, 66% were resistance to ceftriaxone, followed by ceftazidime with 48% and approximately 8% were ESBL positive. None of the isolates were resistant to the carbapenems tested. As for the ARGs profile, were found both 12,70% of *bla*SHV and *bla*TEM among the isolates. It is therefore important to study this threat in order to raise awareness among the population of the importance of prevention and the proper use of antibiotics and since the inappropriate disposal of waste and antimicrobials in water potentiates the spread of resistance to antimicrobials.

## Resumo

### RESISTÊNCIA AOS ANTIMICROBIANOS ENTRE ISOLADOS DE *ESCHERICHIA COLI* EM AMOSTRAS HÍDRICAS DO RIO DO COBRE, SALVADOR, BRAZIL

A falta de saneamento tem uma forte influência na contaminação fecal em águas superficiais urbanas e contribui para aumentar e propagar a resistência aos antibióticos no ecossistema. Este estudo teve como objetivo descrever a ocorrência de bactérias e genes resistentes a antibióticos num sistema fluvial de uma área urbana no Brasil. Amostras de água foram coletadas de 7 pontos de amostragem diferentes do rio Cobre e analisadas para bactérias resistentes aos antibióticos. Os isolados foram identificados por MALDI-TOF<sup>®</sup> e o perfil do antibiograma por VITEK<sup>®</sup>. A presença de genes de resistência aos antibióticos (ARGs) foi estudada por meio da reação em cadeia da polimerase (PCR) convencional. Foram observados ARGs e bactérias resistentes aos antibióticos em todos os locais de estudo. Em 126 isolados de *Escherichia coli* de amostras ambientais revelaram que 59% dos isolados eram resistentes à ciprofloxacina, 44% à amoxicilina/ácido clavulânico e 42% ao aztreonam. Para o grupo

cefalosporina, 66% apresentaram resistência à ceftriaxona, seguido por ceftazidima com 48% e aproximadamente 8% foram ESBL positivos. Nenhum dos isolados foi resistente aos carbapenêmicos testados. Quanto ao perfil dos ARGs, foram encontrados 12,70% de blaSHV e blaTEM entre os isolados. É portanto importante estudar esta problemática de forma a sensibilizar a população sobre a importância da prevenção e do uso adequado de antibióticos e uma vez que a eliminação inadequada de resíduos e antimicrobianos na água potencializa a propagação da resistência aos antimicrobianos.

## Introduction

Antimicrobial resistance is the ability of a microorganism to stop a particular antimicrobial agent from acting on it, thus resulting in ineffective treatments and persistent infections (WHO, 2012). According to the Pan American Health Organization (PAHO, 2017), the excessive and inappropriate use of these substances in humans and animals has been the biggest cause of the spread of resistant bacteria, through water, food and other environmental sources (PAHO, 2017). For many years, the multidrug resistance of bacterial isolates has been a concern for human public health, as well as animal health and the environment (FIOCRUZ, 2019). This becomes a more delicate problem when multidrug-resistant bacteria have a clear ability to transfer resistance genes inter and intra species (ROSSI, 2011). Due to poor sanitation systems, domestic and hospital waste discharged into water collections contribute to these bacteria, especially enterobacteria, being found and disseminated in the environment, in individuals in communities, in animals and in hospitals (BARTLEY *et al*, 2019).

Enterobacteria are a wide variety of Gram-negative bacteria belonging to the Enterobacterales family (KANG E. *et al*, 2018). These microorganisms are often found in the gastrointestinal tract of humans and other animals but can also be found in environments such as soil, water and others (SOUZA *et al*, 2016). Some species of enterobacteria are pathogenic to humans and animals, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*. These bacteria can cause numerous infections, from urinary tract infections to more serious ones such as pneumonia, meningitis and sepsis (JENKINS *et al*, 2017)

In addition, resistance to carbapenems has been reported in some strains of *E. coli*, which are considered 'last resort' antibiotics used to treat infections resistant to other classes of antibiotics (MELETIS, 2016). It is therefore extremely important to study this threat in order to

apply effective control strategies against these infections and to raise awareness among the population of the importance of prevention and the proper use of antibiotics since the inappropriate disposal of waste and antimicrobials in water potentiates the spread of resistance to antimicrobials.

## **Methodology**

### Study site

The Cobre river Rio is approximately 16 km long and is located in the city of Salvador, Bahia, Brazil. Although it is less well known compared to other rivers in the region, it plays a significant role in the local hydrographic system. It runs through urban and rural areas of Salvador, having its source in the Paripe district and flowing through various districts of the city with large demographic density. Despite facing problems of pollution, environmental degradation and siltation in some areas, the river still has relevance for local ecology, serving as a habitat for some species of animals and plants (figure 1).

The seven points selected in this study were the same as described in 2009 in the book "*O caminho das águas em Salvador: bacias hidrográficas, bairros e fontes*" (Universidade Federal da Bahia, Salvador, BA - 2010) (SANTOS *et al.*, 2010), an important book that describes the route of river systems in Salvador and how fresh water disappears in an inverse relationship to the intensity of the urbanization process (Figure 1).

### Water sampling

Water samples were collected from 7 points of the Cobre River every 3 months (Figure 2), from October 2021 to October 2022. At each time point, 400 mL of water was collected in sterile glass vials at a depth of approximately 30 cm below the surface. The vials were transported in thermal boxes with ice until microbiological analysis.

### Microbiological analyses

100  $\mu$ L of the collected water was plated on MacConkey Agar (Merck, Darmstadt, Germany) containing 2 $\mu$ g/mL of cefotaxime (Sigma-Aldrich, USA) for cephalosporin-



resistance screening bacteria (modified from Montezzi et al). Because of the high density of organic contamination, serial dilutions of water (1:10, 1:100 and 1:1000) were prepared and incubated for 24 h at  $36 \pm 2$  °C. Colonies with morphological characteristics suggestive of *Echerichia coli* were inoculated on triple sugar iron (TSI) agar (Neogen, Lansing, Michigan, USA) to determine the fermentation ability. All glucose-fermenting bacteria isolated on TSI were re-isolated on tryptic soy agar (TSA) (Neogen, Lansing, MI, USA) and routed for identification. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF<sup>®</sup>) (VITEK-MS<sup>®</sup>, Biomérieux, France) was employed to identify bacterial species. The AMR susceptibility profile was determined using the VITEK-2<sup>®</sup> automated system for Enterobacteriaceae (Biomérieux, France). Subsequently, bacterial isolates were stored in the tryptic soy broth medium supplemented with glycerol (20%) at  $-80$  °C for further analysis.

#### Detection of antimicrobial-resistant bacteria genes

*E. coli* isolates were re-cultured on TSA media for 18–24 h at  $36 \pm 1$ °C, and colonies ( $\pm 5$ ) were resuspended in 100 $\mu$ L of sterile distilled water for DNA extraction. Each isolate was incubated at 95°C for 5 min and then centrifuged at 12,000 rpm for 2 min. The supernatant was transferred to another cryotube and stored at  $-20$  °C until use.

AMR identification was performed using the conventional polymerase chain reaction (PCR) method using TopTaq Master Mix<sup>®</sup> (Qiagen, USA), in accordance with several different protocols for beta-lactamases genes target (*blaSHV*, *blaTEM*) and carbapenemases (*blaKPC-1*, *blaKPC-4*, *blaVIM-1*, *blaVIM-2*, *blaNDM*, *blaBKC-1*, *blaOXA-23*,) in accordance with the following protocols DALENNE C. et al (2010), VAN DER ZWALUW, K. et al (2015), WOODFORD, Neil et al. (2006), NICOLETTI A. G. et al. (2015).

#### Data analysis

Events were described using frequencies and measures of central tendency (mean, median) and dispersion (standard deviation). To compare categorical variables, the Chi-square test (corrected by Yates) or Fisher's exact test was used when recommended; and for continuous variables, the Kruskal-Wallis non-parametric test was used. The odds ratio (OR) was calculated using the cross-product method. In all statistical tests, a significance level of 5% ( $p < 0.05$ ) was adopted.

## Results

In this study, 126 *Escherichia coli* isolates from environmental samples from the Rio do Cobre were investigated to observe and determine their resistance to commonly used antimicrobial agents (Graphic 1). The results revealed a certain level of resistance in this hydrographic basin. 44% of the isolates were resistant to amoxicillin/clavulanic acid and 42% to aztreonam. For the cephalosporin group resistance, it was observed that the isolates were more resistant to ceftriaxone, approximately 66%, followed by ceftazidime with 48%, with only 4.2%. None of the isolates were resistant to the carbapenems tested, ertapenem and meropenem. However, it was observed that approximately 8% were ESBL positive. The resistance of the fluoroquinolone, ciprofloxacin, was observed in approximately 59% of the *E. coli* isolates in this study.

As for the antimicrobial resistance (AMR) profile genes, both 12,70% of *blaSHV* and *blaTEM* among the isolates. The *blaOXA-1-LIKE* and *blaVIM* genes were found, with rates of 9.52% and 8.73%, respectively. The *blaKPC-1* gene was observed in a percentage of 1.58%, while the other genes, such as *blaIMP*, *blaGES*, *blaOXA23*, *blaNDM* and *blaBKC-1*, were not detected in any isolate tested in this study (Graphic 4). The most frequently identified profile gene combination was *blaTEM* + *blaSHV*, present in 6.35% of cases (8 out of 126). Three other AMR profile combinations were found, such as *blaOXA-1-LIKE* + *blaKPC-1*, ESBL + *blaOXA-1-LIKE*, and *blaOXA-1-LIKE* + *blaVIM*, with a percentage observed of 0.79% in each case (1 of 126 each)

## Discussion

In this study, we found resistant *E. coli* isolates in all 7 river collection points. Overall, our phenotypic analysis revealed that the most frequent antimicrobial resistance was to

cephalosporin group resistance with approximately 66% and from that 7,94% were ESBL-positive. Although cephalosporins were found in different aquatic matrices, they all had sewage and wastewater in common as the main reasons for positive findings (RIBEIRO *et al*, 2018). The presence of ESBL-positive enterobacteria is associated with high AMR dissemination and, consequently, is frequently related to healthcare problems and many community-acquired infections (FURLAN, 2018). The ESBL-positive results were less than the findings for this group of antibiotics compared to the previous study by Bartley *et al.*, with rates of 89%, in an urban lake in the same hydrographic basin as Cobre river (BARTLEY *et al*, 2019).

The analysis revealed that fluoroquinolone resistance, specifically to ciprofloxacin, was identified in approximately 59% of the *E. coli* isolates in this study. This resistance rate represents an increase of almost 50% compared to a previous study carried out in communities in Salvador, Bahia, in 2010, (BARBERINO, *et al* 2010). This remarkable rise in resistance to ciprofloxacin among the *E. coli* strains in our study highlights the importance of the disposal of antibiotics in water as a great determinant to find higher rates of these drugs. This group of drugs is widely used and has excessive consumption in the treatment of urinary tract infections, which may be related to the high rate of resistance and presence of this drug in the water (CONTE *et al.* 2017). Furthermore, this group of drugs is also widely used in aquaculture or animal husbandry, and reinforces the cycle of animal, human and environmental dissemination (JACOBY, 2005).

As for the resistance genes, the genes that were most frequently identified were *blaSHV* and *blaTEM*, while the *blaKPC-1* genes were less common. These results differ substantially from a previous study based on the urban watershed of Dique do Cabrito (BARTLEY, 2019). In that previous study, carbapenem genes such as *blaOXA-48*, *blaKPC-1* and *blaVIM-2* were found in large numbers, while the *blaTEM* gene was only occasionally detected. Our hypothesis for this phenomenon is that, possibly because it is a closed lagoon, multidrug-resistant bacteria may be more concentrated than in open basins such as Cobre river.

Regarding the frequency of the genes presented, it was observed that the resistance of beta-lactam bacteria was identified in a higher proportion (53.17%), corroborating the findings of a previous study that also shows that a plausible explanation for this could be their remarkable ability to spread, often facilitated by plasmids (MORETTO *et al.*, 2021).

Lastly, our findings indicate that high rates of antimicrobial resistance may be associated with a lack of access to appropriate basic sanitation, considering that the watershed selected for the research is densely populated by areas known as 'informal settlements', communities established in an unregulated manner by individuals with low social status (ESSACK, 2021). In view of this study, it can be concluded that antimicrobial resistance bacteria is found in surface waters in an urban river in Salvador and it's a reservoir of AMR genes. To mitigate this threat, it is crucial to promote the responsible use of antimicrobials and raise public awareness about the importance of prevention and the appropriate use of antibiotics and the risks of being in contact with contaminated water.

## Figures and tables

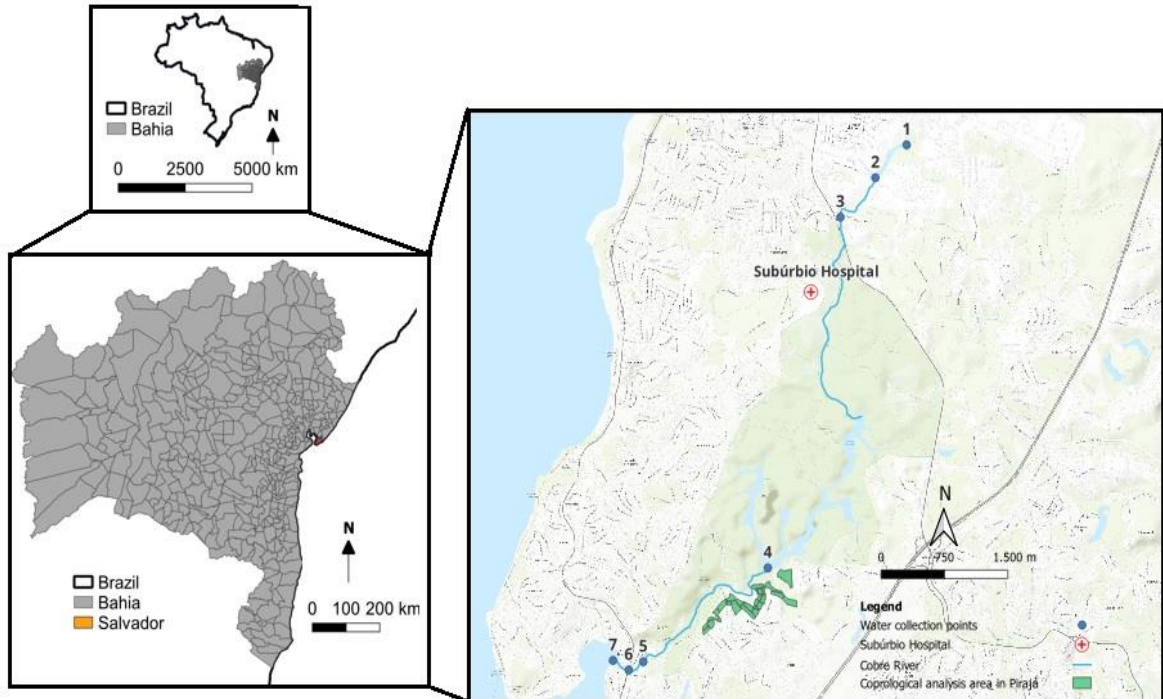


Figure 1 - Cobre river - study site in Salvador

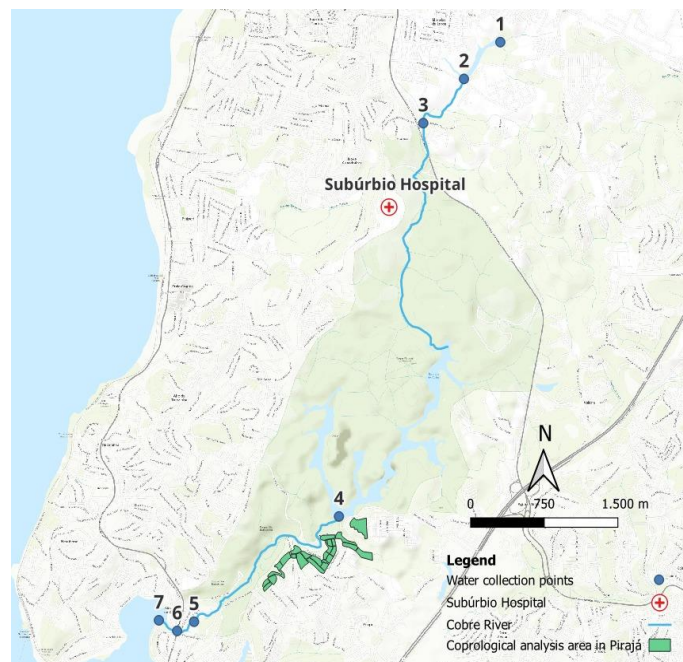
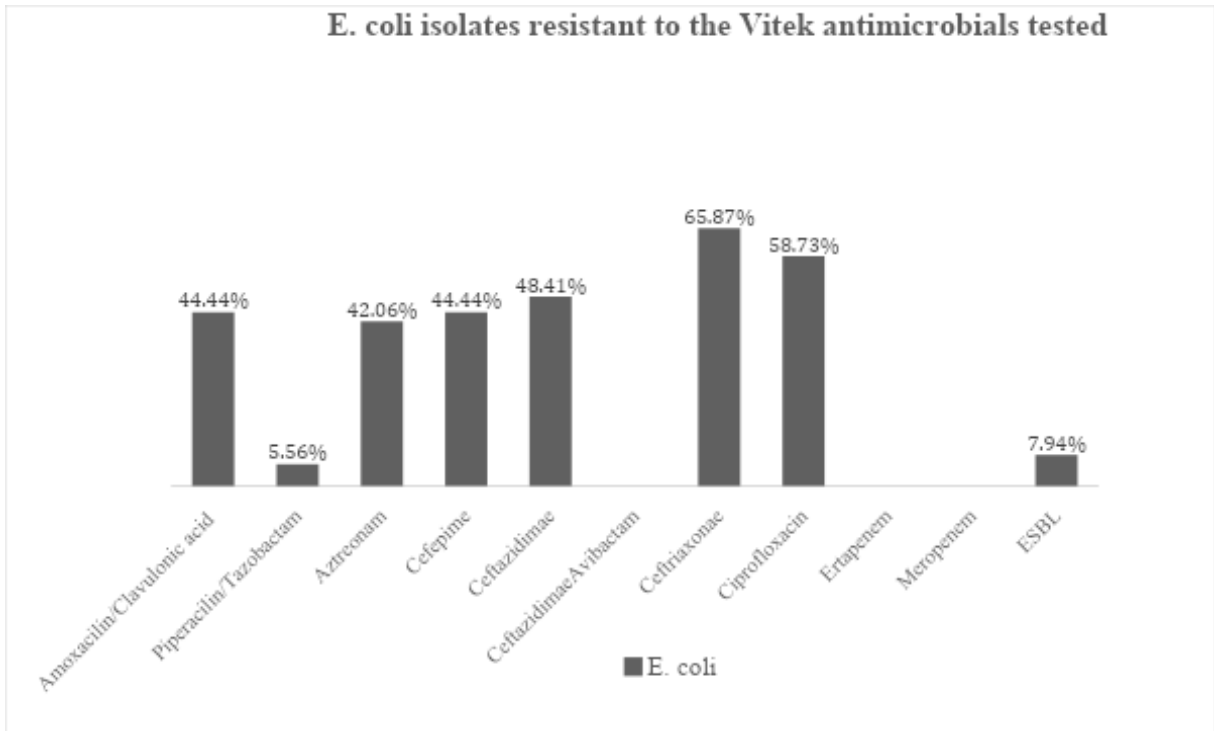
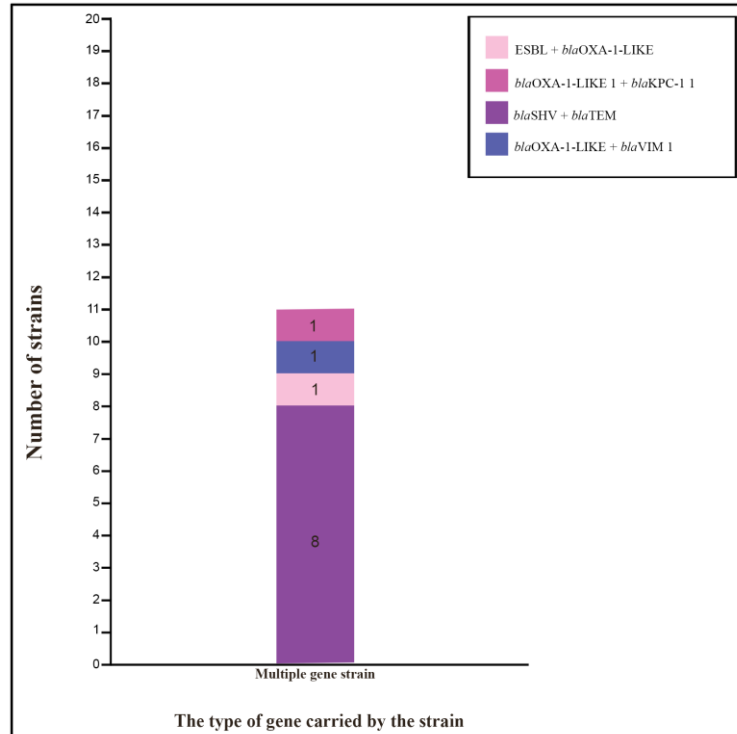


Figure 2 - Collection points of Cobre river in the study



Graphic 1 - AMR susceptibility profile in *E. coli* isolates



Graphic 2 - AMR genes resistance profile in *E. coli* isolates

<i>Beta-lactamase genes</i>	<i>No. positive strain</i>	<i>%</i>
ESBL	10	7,94
<i>bla</i> SHV	16	12,70
<i>bla</i> OXA-1-LIKE	12	9,52
<i>bla</i> VIM	11	8,73
<i>bla</i> TEM	16	12,70
<i>bla</i> KPC-1	2	1,58
<i>bla</i> IMP	0	0
<i>bla</i> NDM	0	0
<i>bla</i> BKC-1	0	0
<i>bla</i> OXA-23	0	0

*Table 1 - AMR resistance profile in E. coli isolates*

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