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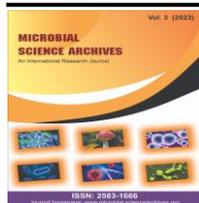
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Research Article

Relative abundance and diversity of extended-spectrum beta-lactamase-producing Enterobacteria in some aquatic environments in an urbanized area in DR Congo

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Abstract

ESBL-producing enterobacteria have a significant impact on public health as they provide antibiotic resistance. Little is known about the relative diversity in various types of biotopes in the same geographical area. This study aimed to determine their diversity and abundance in 3 different aquatic environments. Samples from wells, river water, and hospital wastewater were analyzed. ESBL-producing enterobacteria isolated were identified using double-disk synergy test method based on enzymatic tests. Results showed that the abundance of total enterobacteria in log (CFU/100 μ L) varied from 0.60 to 2.10 in wells, from 0.79 to 5.73 in rivers, and from 1.58 to 6.52 in hospital wastewater. That of ESBL-producing enterobacteria reached 6.23 in hospital wastewater, 3.08 in river samples and 2.04 in wells. Their relative abundances compared to all of the isolated enterobacteria varied from 0.34 to 4.55% in wells, from 0 to 97.5% in rivers and from 0.73 to 61.29% in hospital wastewater. Their abundances and diversity varied significantly between hospital wastewater and the 2 other types of samples analyzed ($P < 0.05$). ESBL-producing enterobacteria identified included *Escherichia coli*, *Ewingelia americana*, *Erwina spp*, *Citrobacter freundii*, *Edwardsiella spp*, *Klebsiella ozaenae*, *Shigella spp*, *Enterobacter aerogenes*, *E. agglomerans*, *Citrobacter diviersys*, *Proteus mirabilis*, *Serratia fonticola*, *Serratia ficana*, *Moellerella wisconsensis*, *Klebsiella rhinoscleromatis* and *Providencia rettgeri*. Among these, 12 were identified in hospital wastewater, 6 in river water and 4 in wells. The Shannon and Weaver diversity index showed an uneven distribution of ESBL-producing enterobacteria species and the dominance of a few species. Most species in wells and rivers were identified in hospital wastewater.

Keywords: ESBL-Producing Enterobacteria, Abundance, Diversity, Wells, River Water, Hospital Wastewater.

Introduction

Beta-lactamases are enzymes produced by bacteria that provide resistance to beta-lactam antibiotics. They break down the antibiotics' structure and destroy some commonly used antibiotics, including penicillins and cephalosporins, and make these drugs ineffective for treating infections (Elhani, 2012; Akhtar et al., 2022). Beta-lactam antibiotics share a common

element in their molecular structure, a central four-membered ring, the β -lactam. Beta-lactamases hydrolyse this cycle, thus deactivating the antibiotic properties of the molecule. Members of extended-spectrum beta-lactamase (ESBL) family commonly express beta-lactamases which confer resistance to expanded-spectrum (extended-spectrum) cephalosporins (Nicolas-Chanoine, . The prevalence of ESBL-producing bacteria has been gradually increasing in the general

population and it varies from one areas to another (Spadafino et al., 2014; Symanzik et al., 2022).

Enterobacteriaceae of the intestinal flora producing ESBLs and contained in waste water can sometimes be discharged into the environment without any prior treatment. Following precipitation, the microbial loads of this waste water can thus pollute the soil, subsoil, groundwater and surface water. During their transfer and during their stays in these various media which have different abiotic properties, progressive or regressive modifications can occur. Contaminated surface waters could be the potential site of transmission of resistance to humans through contact at numerous interfaces (Manaia, 2017; Surette & Wright, 2017). The different microbiomes in terrestrial ecology interact directly or indirectly at different interfaces (Roberts, 2018).

The presence of ESBL-producing Enterobacteriaceae has been reported in several ecosystems (Peng et al., 2019; Andrade et al., 2020), each having its own intrinsic characteristics although certain properties are common. They are found in hospitals, in food production chains or in various types of aquatic environments (Freitas et al., 2019; Montero et al., 2021). Hospital and confined animal husbandry effluents are considered areas at risk of contamination (Karkman et al., 2019; Duan et al., 2019). Thus, rivers, lakes, sediments, and groundwater-collecting effluents carry multi-resistant germs (Peng et al., 2019; Vikesland et al., 2019; Andrade et al., 2020).

Seasonal changes in various regions of the globe sometimes lead to structural and physiological changes in microorganisms, as well as their relationships with other biotic or abiotic components of the environment (Thornton et al., 2020; Andrade et al., 2020). Although a lot of information is available on the resistance of these ESBL-producing Enterobacteriaceae, little is known about their relative diversity in various types of biotopes in the same geographical area. Little is also known about the impact of seasonal changes on their abundance dynamics, cell diversity, as well as on the production of ESBLs in various aquatic systems. The present study aims to evaluate in different types of aquatic environments (hospital waste water, river water and groundwater) in an urban area in the Democratic Republic of Congo (DR Congo), (Central Africa), the abundance dynamics and the diversity of enterobacteria producing ESBLs during the long rainy season and the long dry season.

Materials and methods

Study area and sampling sites

The study was carried out in the city of Butembo, in the east of the DR Congo during the period from August 2022 to February 2023. This period covers the two main seasons of the locality, which are the rainy season (August-December) and the dry season (January and February). Samples from 3 well water points, 3 hospital waste water and 3 sites on the Kimemi

River (which crosses the city) were analyzed. The number of sampling points was 9. Fig. 1 shows the locations of study sites in the city of Butembo. The sampling frequency was monthly.

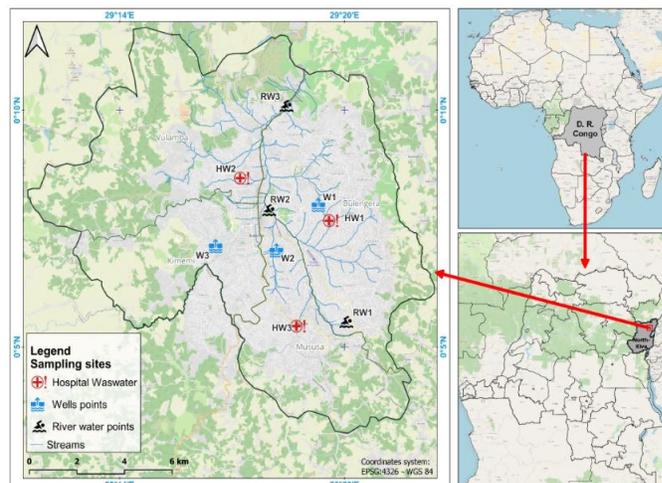


Fig. 1 Sampling sites in Butembo town (HW: Hospital waste water; W: wells points; RW: River water points)

Meteorological parameters during the study period

The meteorological data during the sampling period were obtained from the Butembo airport station. During this sampling period, the rainfall varied from 3,37 to 286,3 mm/day. The air temperature and the air relative humidity varied from 11,8 to 24,65°C and from 87,52 to 94 % respectively. The insolation varied from 573,5 to 796,2 Kw-h/m²/day. The sunshine varied from 55h04' to 87h45'/day. The temporal variation of these data is presented in Fig. 2.

Water sampling

The study was carried out during the period from August 2022 to February 2023. This period covers the two main seasons of the locality, which are the rainy season (August-December) and the dry season (January and February). The sampling frequency was monthly. Water samples were taken in sterile 500 mL glass bottles for microbiological analyses, and clean 1000 mL polyethylene bottles for physicochemical analyses. All the samples were then placed in an isothermal cold container and immediately brought back to the laboratory for analysis.

Bacteriological analyses

Isolation of total mesophilic aerobic heterotrophic bacteria (MAHB) and of enterobacteria

After homogenization of the water samples, followed by decimal dilutions, the total mesophilic aerobic heterotrophic bacteria (MAHB) were isolated on standard agar medium contained in petri dishes using plate count method. The

colonies forming units (CFU) were then counted after 3-5 days of incubation at laboratory temperature (25 ± 2 °C). This was done using a STUART SCIENTIFIC colony counter and the results were expressed as $\log(\text{CFU}/100\mu\text{L})$.

For the isolation of the total enterobacteria, water samples after homogenization followed or not by decimal dilutions, were spread on the surface of Mac Conkey agar (Titan Biotech LTD: W/CV, NaCl, 0.15% Bile salts and 1% Lactose) contained into petri dishes and then incubated at 37°C for 18 to 24 hours (Denis, 2016). The macroscopic observation of the enterobacterial CFUs consisted of examining the cultural characteristics of the bacteria expressed in colonies. These include the color, size, contours and surface configuration of the colony. Colonies were counted using a STUART SCIENTIFIC colony counter indicated above. The results were also expressed as $\log(\text{CFU}/100\mu\text{L})$. After purification of the strains, their identification was carried out by the enzymatic method (Denis, 2016).

Detection of ESBL-producing enterobacteria

ESBL-producing enterobacteria have been highlighted by the search for synergy between clavulanic acid and 3rd generation cephalosporins. Enzymes are highlighted by the disc method which consists of looking for an image of champagne cork synergy between an antibiotic disc which contains a β -lactamase inhibitor and the 3rd generation cephalosporin discs. The antibiotic discs used were ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), Amoxicillin/Clavulanic Acid (20/10 μg) and aztreonam (30 μg). The production of ESBLs results in the appearance of an image of "Champagne cork" synergy between the discs of Amoxicillin/Clavulanic Acid and 3rd generation cephalosporins (Chaturvedi et al., 2021).

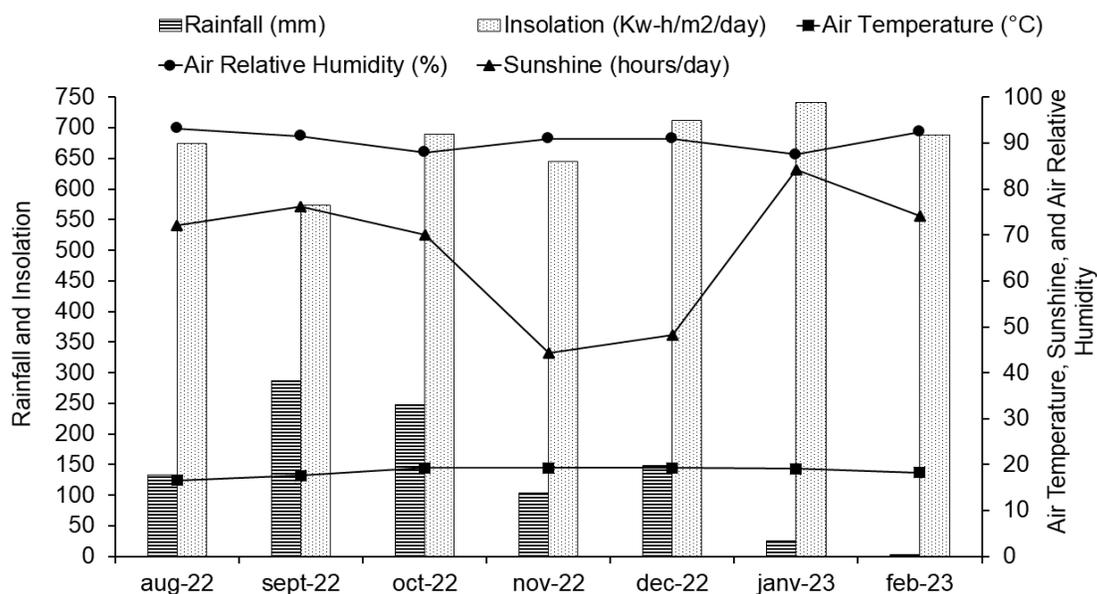


Fig. 2 Temporal variations of rainfall, insolation, sunshine, air relative humidity and air temperature at Butembo during the sampling period (from August 2022 to February 2023)

Physicochemical analyses

The parameters considered in this study included water pH, electrical conductivity, turbidity, nitrite and nitrate concentrations. Some of them (water pH and electrical conductivity) were measured in the field using a multi-parameter HACH/POCKET PRO⁺ and the others were measured in the laboratory using a spectrophotometer CECIL CE1011. This was done according to the recommended techniques (Rodier et al., 2016).

Data analysis

The spatio-temporal variations of the considered parameters were illustrated using curves and histograms. The relationships and comparisons between these parameters were evaluated using Spearman's correlation and the Kruskal Wallis tests respectively. The diversity of the identified ESBL-producing enterobacteria was assessed by the Shannon and Weaver diversity index (Boudem Tsane et al., 2021).

Results

Spatio-temporal variation in the abundance of the total MAHB and total Enterobacteria

During the study, the abundance of MAHB fluctuated between 1.99 and 3.89 (log CFU/100µL) in the wells, between 1.23 and 14.35 (log CFU/100µL) in the water from river, and between 5.43 and 8.75 (log CFU/100µL) in hospital waste water (Fig. 3). The highest abundances were overall recorded in September. The lowest abundance was recorded during December in wells and hospital waste water, and during October in river water (Fig. 3).

The abundance of total enterobacteria varied between 0.60 and 2.0 (log CFU/100µL) in wells, between 0.78 and 5.73 (log CFU/100µL) in river water, and between 1.58 and 6.60 (log CFU/100µL) in hospital waste water (Fig. 4). The highest abundances were recorded during August and January in wells, during February in river waters, and during December in hospital waste water. The lowest abundances were recorded during October in wells, and during August and January in rivers and hospital waste water (Fig. 4).

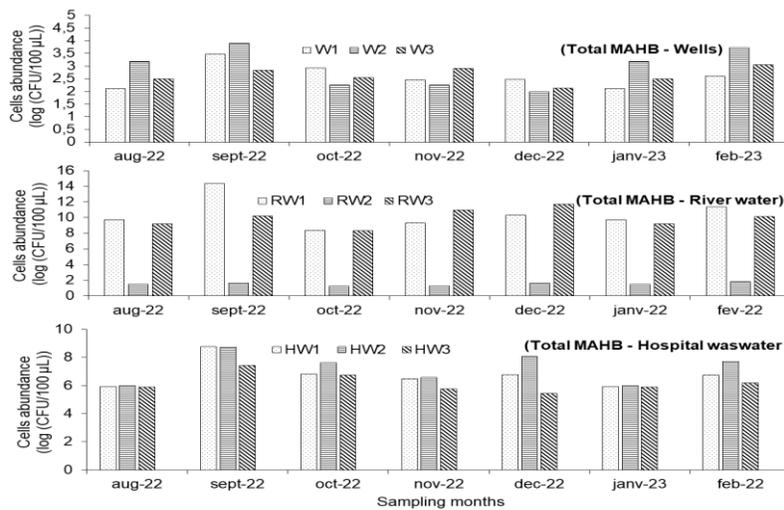


Fig. 3 Abundance of the total mesophilic aerobic heterotrophic bacteria (MAHB) in each wells, river water and hospital waste water analysed with respect to each sampling month

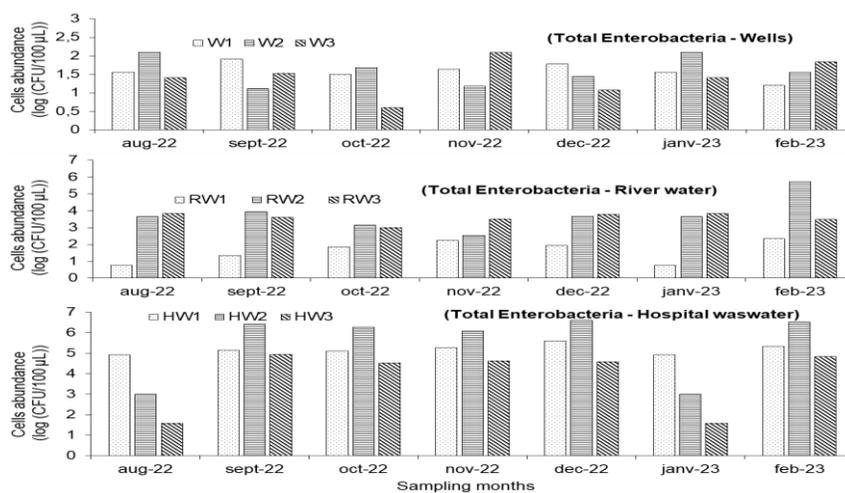


Fig. 4 Abundance of the total enterobacteria in each wells, river water and hospital waste water analysed with respect to each sampling month

Abundance and diversity of the ESBL-producing enterobacteria

The highest abundances of ESBL-producing enterobacteria reached 2.05 (log CFU/100 μ L) in wells, 5.73 (log CFU/100 μ L) in river waters, and 6.45 (log CFU/100 μ L) in hospital wastewater. These ESBL-producing enterobacteria were sometimes rare in some sites for example the RW1 of river, and well water points (Fig. 5).

For all well water points analyzed, 4 species of ESBL-producing enterobacteria were identified. They are *Citrobacter freundii*, *Enterobacter aerogenes*, *Yersinia pseudotuberculosis* and *Moellerella wisconsensis* (Fig. 5). In the river water samples, 6 species of ESBL-producing enterobacteria were identified. They are *Escherichia coli*, *Ewingelia americana*, *Citrobacter freundii*, *Proteus mirabilis*, *Providencia rettgeri* and *Enterobacter aerogenes* (Fig. 5). In hospital waste water samples, 12 species were identified. They are *Escherichia coli*, *Ewingelia americana*, *Erwina spp*, *Edwarsiella spp*, *Klebsiella ozaena*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Citrobacter diversys*, *Serratia fonticola*, *Moellerella wisconsensis*, *Klebsiella rhinoscleromatis* and *Cedecea lapagei* (Fig. 5).

In each of the 9 study sites, the Shannon and Weaver diversity index has been calculated. The value was 0. It thus reflects an uneven distribution of ESBL-producing enterobacteria species and the dominance of a few species. In wells, *Enterobacter aerogenes* is dominant. In river water, *Ewingelia americana* dominated. In hospital waste water *Klebsiella rhinoscleromatis* and *Ewingelia americana* dominated.

In sampled sites, the relative abundances of enterobacteria compared to MAHB, as well as those of ESBL-producing enterobacteria compared to total enterobacteria, were calculated. These relative abundances are presented in Table 1. It appears that the relative abundances of enterobacteria varied from 2.35 to 6.18% in wells, from 0.76 to 6.01% in river water, and from 0.21 to 1.71% in hospital waste water. Compared to total enterobacteria, the relative abundances of ESBL-producing enterobacteria varied from 0.34 to 4.55% in wells, from 0 to 97.50% in river water, and from 45.51 to 61.29% in hospital waste water.

Physicochemical parameters of the water samples

The pH varied from 5.02 to 7.32 in wells, from 5.28 to 7.66 in river water, and from 5.29 to 7.13 in hospital waste waters (Fig. 6). The highest concentrations of nitrites and nitrates reached respectively 4.56 and 5.86 mg/L in wells, 24.41 and 17.39 mg/L in river water, and 4.53 and 10.92 mg/L in hospital waste waters. The highest value of turbidity was 9.09 NTU in wells, 41.14 NTU in river water and 47.5 NTU in hospital waste water. Wells and river water have sometimes been clear. The electrical conductivity fluctuated between 150 and 2630 μ S/cm in wells, between 240 and 1630 μ S/cm in the

river, and between 340 and 3130 μ S/cm in hospital waste waters. Overall, the values of these abiotic parameters underwent relative spatiotemporal fluctuations (Fig. 6-7).

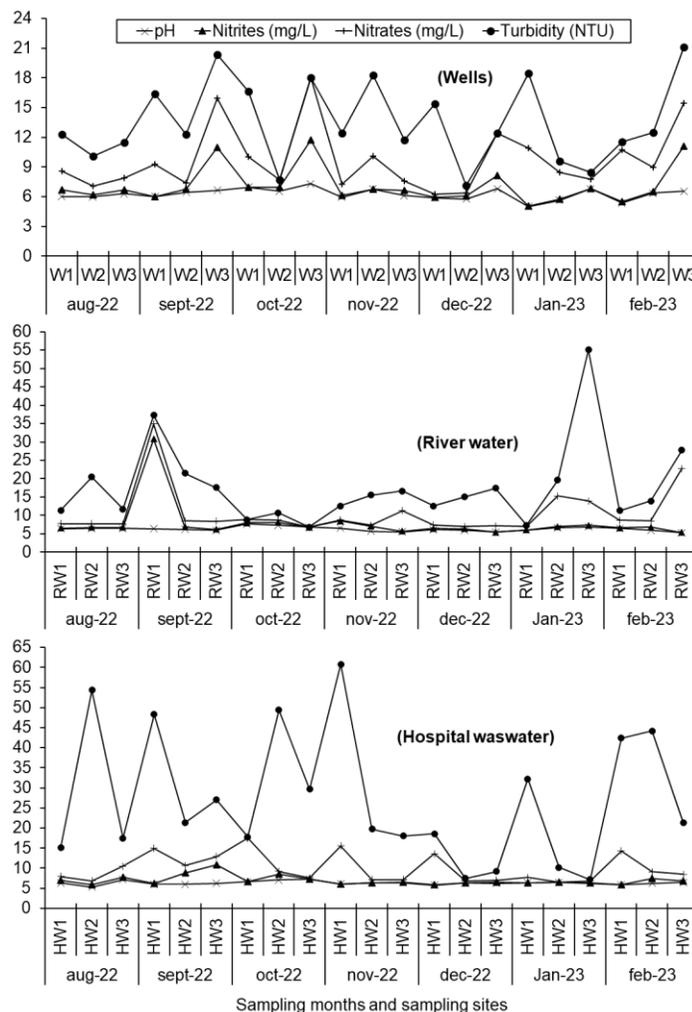


Fig. 6 Temporal variation of the pH, nitrates, nitrites and turbidity values of the water samples analysed with respect to each sampling month

Correlations and comparisons between the considered parameters

In each study site, the correlations were assessed using Spearman's "r" test between the bacteriological and the water physicochemical parameters on the one hand, and between the bacteriological and meteorological parameters on the other hand. Overall, this results in weak and non-significant correlations ($P > 0.05$). The same observations were noted about the correlations between MAHB and enterobacteria.

The comparisons of parameters were made using the Kruskal Wallis (H) test. As a result, the concentrations of ESBL-producing enterobacteria in hospital waste water differ significantly from those in groundwater as well as river water

($P < 0.001$). However, wells do not differ significantly from river water ($P > 0.05$). In addition, the abundances of ESBL-producing enterobacteria did not differ significantly between

the 2 seasons of the study (between the long dry season and the long rainy season) ($P > 0.05$).

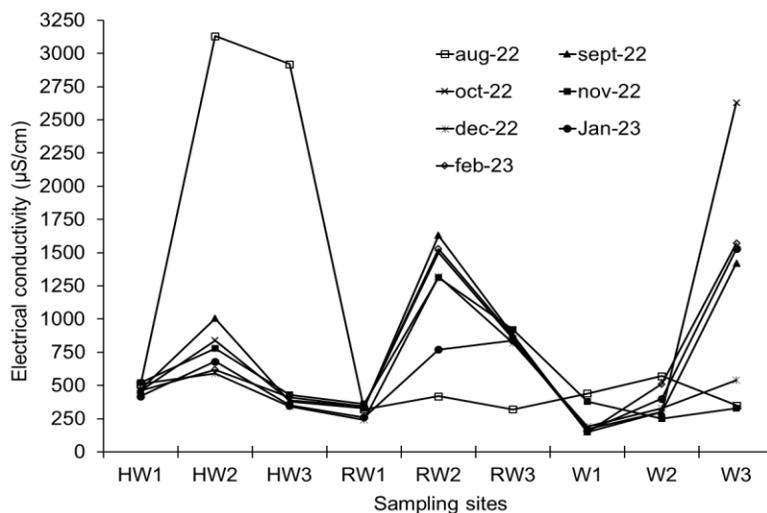


Fig. 7 Temporal variation of the electrical conductivity values of the water samples analysed with respect to each sampling site

Results showed that abundance of total MAHB, total enterobacteria and total ESBL-producing enterobacteria are relatively low at RW1 which is located upstream. It has been indicated that surface water is highly impacted by human activities as it receives contaminants from different sources (Conte et al., 2017; Cho et al., 2023). Other river water

sampling points would be exposed to different anthropogenic impacts. It is also indicated that rivers with higher level of anthropogenic pollution tend to contain more number of ESBL-producing bacteria (Conte et al., 2017; Cho et al., 2023).

Table 1. Relative abundance (percentage) of the total enterobacteria and total ESBL-producing enterobacteria

| Considered bacteria in the abundance ratio | Sampling sites and the ratio values (percentage) | | | | | | | | |
|--|--|------|------|------|------|-------|-------|-------|-------|
| | W1 | W2 | W3 | RW1 | RW2 | RW3 | HW1 | HW2 | HW3 |
| Total enterobacteria/Total MAHB | 6.18 | 2.35 | 3.75 | 6.01 | 7.74 | 0.76 | 0.21 | 1.71 | 0.73 |
| Total ESBL-producing enterobacteria/ Total enterobacteria | 4.55 | 2.50 | 0.34 | 0.00 | 97.5 | 26.27 | 45.51 | 61.29 | 50.00 |

It has been noted that in the 3 types of aquatic ecosystems considered, the abundance of ESBL-producing enterobacteria underwent spatiotemporal variations. This has also been noted by Dantas Palmeira & Neto Ferreira, (2020). In addition, these authors indicate that for a given species, the beta-lactamase enzymes produced also vary with respect to the country, as well as with the strains properties. They noted that some of the strains they isolated (*Enterobacter cloacae*, *E. aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *K. ozaenae*, *K. ozaenae*, *K. oxytoca*, *Serratia enterica*, *S. marcescens*, *Citrobacter freundii*, *C. koseri*, *C. brakii*, *C. brakii*, *C. youngae*, *Proteus mirabilis*, *P. vulgaris*, *Salmonella* sp, *Providencia* spp and *Shigella* spp) can be commensal and others can be pathogen. Some strains produced many beta-

lactamase enzymes. Conversely, a given beta-lactamase enzyme in some cases was produced by different bacterial species from different parts of the world (Dantas Palmeira & Neto Ferreira, 2020).

The abundance of ESBL-producing enterobacteria registered varied with respect to the type of water. According to Chaturvedi et al. (2021), anthropogenically impacted surface waters are an important reservoir for multidrug-resistant bacteria including ESBL-producing microorganisms, and antibiotic-resistant genes, including efflux genes, and heavy metals resistance genes. The resistance could be towards beta-lactams, lincosamides, glycopeptides, cephalosporins, sulphonamide, macrolide, and tetracycline, among others. Cho

et al. (2023) reported that waste water treatment plants are considered hotspots for the emergence and dissemination of antimicrobial resistance as they receive antibiotic-resistant bacteria, antibiotic resistance genes, and antibiotic residues, along with waste water from various sources, such as households, hospitals, and industries.

High concentrations of bacteria in the nutrient-rich environments within waste water create a favorable environment for the selection of antibiotic-resistant bacteria and the exchange of antibiotic resistance genes. Significant changes may however appear in the quantity and quality of antibiotic-resistance genes during their transport and spreading in the aquatic environments (An et al., 2018). The ability of environmental bacteria to successfully transfer resistance genes via conjugation has also been indicated as well as the emergence of novel antibiotic resistance genes in the absence of external influences, and the capacity of environmental bacteria to disseminate antibiotic resistance genes via mobile genetic elements which include the integrons, plasmids, insertion sequences and transposons (Hooban et al., 2020).

The spreading of the ESBL-producing bacteria in the environment is often linked to many factors. Travel has been strongly associated with ESBL colonization (Arcilla et al., 2020; Larramendy et al., 2020). Other risk factors included previous hospitalization although wimming in freshwater and many socioeconomic and behavioral factors have been reported to increase the likelihood of exposure to some ESBL-producing enterobacteria (Collignon et al., 2018).

Although no significant correlation has been found between the considered biotic and abiotic parameters in this study, Chaturvedi et al. (2021) reported that some abiotic parameters of water such as Biochemical Oxygen Demand and Chemical Oxygen Demand can be positively and significantly correlated to some resistance genes such as *sul1*, *sul2*, *arcB*, *tolC*, *int1* and *int2*. In contrast, other parameters such as the pH and Dissolved oxygen can be negatively and significantly correlated to other resistance genes such as *tolC*, *sul1*, *sul2*, *int1*, *arcA* and *arcB*.

Although the content of organic matter has not been analysed in this study, Miao et al. (2022) working on bacterial community assembly and beta-lactamase genes regulation in a full-scale chlorinated drinking water supply system, noted that the abundance of beta-lactamase genes was associated with bacterial community assembly events. When the heterotrophic plate count was relatively lower, the bacterial community was less affected by the environment. When it was relatively greater, deterministic assembly played an important role and decreasing the organic matter improved the efficiency of beta-lactamase gene control. They concluded that organic matter can affect beta-lactamase genes by shifting the diversity of the microbial community.

The air temperature of the study areas varied from 11.8 to 24.65°C. Although no significant correlation has been noted

between meteorological parameters and the abundance of ESBL-producing enterobacteria, it is indicated that environmental temperature can sometimes impact antibiotic-resistance genes. Ke et al. (2023) noted in fact that bacterial distribution could enrich antibiotic resistance genes in higher temperatures by increasing mobile genetic elements co-occurring with antibiotic resistance genes and diversifying antibiotic resistance gene hosts. In addition, Bock et al. (2022) indicated that an increase in outdoor temperature may be associated with an increase in ESBL-Enterobacteriaceae-incidence, independent of important confounding factors such as antimicrobial consumption. The absence of a significant correlation between the considered physicochemical parameters and the identified ESBL-producing enterobacteria could be due to the confounding factors occurring in each water ecosystem.

Conclusion

The relative abundances of enterobacteria compared to MAHB, as well as those of ESBL-producing enterobacteria compared to total enterobacteria in wells, river and hospital wastewater points considered varied with respect to sampling sites. The physicochemical parameters underwent spatiotemporal variation. The abundance of ESBL-producing enterobacteria has not been impacted by the considered meteorological and water physicochemical factors. ESBL-producing enterobacteria were more abundant in hospital wastewater and less abundant in the river water. They were rare in wells. The 3 aquatic ecosystems were characterized by an uneven distribution of ESBL-producing enterobacteria species and the dominance of a few species. Most ESBL-producing enterobacteria species in wells and rivers were identified in hospital wastewater. Good management of hospital wastewater and river water could improve the bacteriological quality of wells.

Conflict of Interest

The author hereby declares no conflict of interest.

Consent for publication

The author declares that the work has consent for publication.

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