

1 **Multidrug-resistant *Enterobacter* spp. in wastewater and surface water:**
2 **Molecular characterization of β -lactam resistance and metal tolerance**
3 **genes**

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27 **ABSTRACT**

28 Among the ESKAPE group pathogens, *Enterobacter* spp. is an opportunistic Gram-
29 negative bacillus, widely dispersed in the environment, that causes infections. In the
30 present study, samples of hospital wastewater, raw and treated urban wastewater, as well
31 as surface receiving water, were collected to assess the occurrence of multidrug-resistant
32 (MDR) *Enterobacter* spp. A molecular characterization of β -lactam antibiotic resistance
33 and metal tolerance genes was performed. According to identification by MALDI-TOF
34 MS, 14 isolates were obtained: 7 *E. bugandensis*, 5 *E. kobei*, and 2 *E. cloacae*. The
35 isolates showed resistance mainly to β -lactam antibiotics, including those used to treat
36 infections caused by MDR bacteria. Multiple antibiotic resistance index was calculated
37 for all isolates. It allowed verify whether sampling points showed a high risk due to
38 antibiotic resistant *Enterobacter* spp., as well as to determine if the isolates have been in
39 environments with a frequent antibiotic use. Twelve isolates showed β -lactam antibiotic
40 resistance gene, being the *bla_{KPC}* widely detected. Regarding metal tolerance, 13 isolates
41 showed at least two genes that encode metal tolerance mechanisms. Overall, metal
42 tolerance mechanisms to silver, copper, mercury, arsenic and tellurium were found. New
43 data on metal tolerance mechanisms dispersion and antibiotic-resistance characterization
44 of the *E. bugandensis* and *E. kobei* species were here provided. The occurrence of MDR
45 *Enterobacter* spp. in analyzed samples draws attention to an urgent need to put control
46 measures into practice. It also evidences waterborne spread of clinically important
47 antibiotic-resistant bacteria recognized as critical priority pathogens.

48

49 *Keywords:* ESKAPE, antibiotic resistance, carbapenemase, ESBL, wastewater, surface
50 water.

51

52 **1. Introduction**

53 According to the World Health Organization (WHO), antimicrobial resistance
54 (AMR) is recognized among the ten threats to global health by antibiotic resistance (AR).
55 It contributes not only to high mortality rates, but also to economic impacts related to
56 costs for treating infections (WHO, 2019). Only in 2019, antibiotic-resistant bacteria
57 (ARB) were associated to the death of more than 1.2 million people, with bacteria
58 belonging to ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*
59 *pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.)
60 group pathogens as the most frequent etiologic agents (Murray et al., 2022). *Enterobacter*
61 spp. are mobile Gram-negative bacilli part of gut microbiota of animals and humans. They
62 have an opportunistic behavior, which causes serious nosocomial infections highlighting
63 the potential adverse health effects in patients under mechanical ventilation in intensive
64 care units (Davin-Regli et al., 2019).

65 To date, more than 20 species belonging to the genus *Enterobacter* have been
66 identified. Among them, *E. cloacae* and *E. hormaechei* are the most frequently associated
67 with clinical infections. However, recently other species, such as *E. kobei* and *E.*
68 *bugandensis*, have drawn attention for their ability to cause infections and outbreaks
69 (Manandhar et al., 2022; Pati et al., 2018). Due to AmpC β -lactamase expression, these
70 opportunistic pathogens are intrinsically resistant to amoxicillin, ampicillin, cefoxitin and
71 to first generation cephalosporins, while in intra-hospital settings are commonly
72 associated with multidrug-resistant (MDR) phenotype. This means a resistance profile to
73 three or more antibiotics classes, as a consequence of the ability to acquire several mobile
74 genetics elements harboring resistance genes (Davin-Regli et al., 2019; Magiorakos et al.,
75 2012).

76 Carbapenems and 3rd- and 4th-generation cephalosporins are often used to treat
77 infections by MDR *Enterobacter* spp. However, the wide distribution of coding genes for
78 carbapenemases and extended-spectrum β -lactamases (ESBL) in *Enterobacterales*
79 family, including *Enterobacter* spp., compromise the therapy with these antibiotics (Bush
80 and Bradford, 2020). Considering the risk to global health by antibiotic resistance, in 2017
81 the WHO established a priority pathogen list in which new antimicrobials are needed
82 (WHO, 2017). In relation to this, metals such as silver, copper and mercury are promising
83 for bacterial infections treatment due to their antimicrobial potential. Notwithstanding,
84 some metal tolerance mechanisms might compromise their viability as antimicrobial
85 agents. Anyhow, the epidemiological distribution of these tolerance mechanisms remains
86 still unknown (Hobman and Crossman, 2015; Zagui et al., 2021).

87 MDR *Enterobacter* spp. is frequently detected in intra-hospital environments
88 (Boutarfi et al., 2019; Chen et al., 2021; Liao et al., 2022). Special attention has been paid
89 to hospital wastewater, taking into account its potential to harbor ARB and allow its
90 spread to other environments, including urban wastewater and surface water (Hocquet et
91 al., 2016; Zhu et al., 2023). A number of studies around the world have confirmed the
92 presence of ARB in different kinds of aquatic matrices (Kutilova et al., 2021; Kang et al.,
93 2022; Noman et al., 2023). In Brazil, as well as in other developing countries, hospital
94 wastewater treatment is mandatory only for those cities, which do not have urban
95 wastewater treatment plants (UWWTP) (Carraro et al., 2016). On the other hand,
96 allowing discharged of untreated hospital wastewater may favor the pathogens spread
97 through the sewage galleries, increasing the microbial load on the UWWTP. This practice
98 does not guarantee the complete removal of these microorganisms with secondary
99 treatment, as already evidenced by its presence in treated effluents (Manaia et al., 2018;
100 Kumar et al., 2020).

101 The present study was aimed at determining the occurrence of MDR *Enterobacter*
102 spp. in aquatic matrices. Considering the pathogenic potential of that organism for both,
103 humans and animals, the genotype for β -lactam antibiotic resistance and metal tolerance
104 were also investigated.

105

106 **2. Materials and methods**

107 *2.1. Study area and sample collection*

108 Ribeirão Preto city is located at the northeast of São Paulo State, Brazil. It has a
109 large population (>710,000 inhabitants) and many tertiary hospitals (IBGE, 2023). One
110 of them is a sizable university hospital, which covers an area of more than 4 million
111 inhabitants, whose untreated wastewater is disposed into a public collection system
112 according to the current legal regulation RDC 50/2002 (Brazilian Health Regulatory
113 Agency, 2002). The collected urban and hospital wastewater is carried to the UWWTP,
114 which uses activated sludge technology for treatment with no tertiary treatment.
115 Ultimately, the treated wastewater is disposed on an urban stream. In order to evaluate
116 the microbial dispersion in different aquatic matrices, water samples were collected at
117 different locations: the tertiary hospital (untreated wastewater collected at confluence
118 point of the effluents from different sectors of the hospital, such as wards, ambulatories
119 and also laboratories), the UWWTP (raw and treated wastewater), and the urban stream,
120 that receives treated wastewater by UWWTP (upstream and downstream). The upstream
121 samples were collected 350 m away from the treated effluent discharge point into the
122 river, while the downstream samples were collected 300 m away from the effluent
123 discharge point. Water samples of 1 L were collected between March and September 2022
124 and stored according to the Standards Methods for the Examination of Water and
125 Wastewater (APHA/AWWA/WEF, 2017).

126

127 2.2. Sample processing, bacterial isolation and identification

128 Samples were subjected to serial dilution and vacuum filtration through cellulose
129 ester membrane. Subsequently, they were put in Petri dishes containing selective
130 chromogenic medium for isolation of carbapenemase- and ESBL-producing bacteria,
131 according to the manufacturer's instructions (Probac®, Brazil) (Haller et al., 2018). After
132 the incubation period (24 h), colonies with green colors (presumptive for *Enterobacter*
133 spp.) were selected for further analysis. The isolates were grown on MacConkey agar to
134 obtain pure colonies, being stored at -80°C in Brain Heart Infusion broth supplemented
135 with glycerol (20%). The isolates were identified with Matrix-Assisted Laser
136 Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS, Bruker
137 Daltonics, Billerica, MA, USA) (Dingle and Butler-Wu, 2013).

138

139 2.3. Antibiotic susceptibility testing

140 The isolates were tested against 19 antibiotics -belonging to different classes- by
141 disc-diffusion method according to the Brazilian and the European Committees of
142 Antimicrobial Susceptibility Testing (BrCAST, 2022; EUCAST, 2022). Briefly, a
143 suspension of the bacterial isolates in 0.9% NaCl solution was done, similar to a turbidity
144 degree of 0.5 on the McFarland scale. After this, a cotton swab was submerged on the
145 suspension with subsequent seeding on Petri dishes containing Mueller-Hinton Agar.
146 Antibiotic discs (Oxoid) were disposed with automatic dispenser on the plates, proceeded
147 by 24 h incubation period at $35 \pm 1^\circ\text{C}$. The following antibiotics were used: amoxicillin-
148 clavulanate, ampicillin-sulbactam, piperacillin-tazobactam, cefepime, ceftazidime,
149 cefotaxime, ceftazidime, ceftazidime, ceftazidime, ceftazidime, ceftazidime, ceftazidime,
150 levofloxacin, norfloxacin, amikacin, tobramycin, gentamycin, sulfamethoxazole-

151 trimethoprim, and chloramphenicol. *Escherichia coli* ATCC 25922 strain was used as
152 quality control.

153

154 2.4. Multiple antibiotic resistance (MAR) index

155 The MAR index is a numerical representation of the degree to which one bacterial
156 isolate is resistant in relation to all the tested antibiotics. It is widely used to verify high-
157 risk sources for antibiotic resistance. When the index exceeds 0.2, it is considered that the
158 isolated bacterium has previously been in situations in which antibiotics were employed
159 (Krumperman, 1983).

160 MAR index was calculated according to the following equation:

$$161 \text{MAR}_{\text{index}} = \frac{a}{b}$$

162 where a represents the number of antibiotics for which a bacterial isolate exhibits
163 resistance phenotype, being b the total number of tested antibiotics ($n = 19$ antibiotics,
164 see section 2.3) (Krumperman, 1983).

165

166 2.5. Molecular investigation of genes coding for β -lactamase and metal tolerance

167 Genomic DNA (gDNA) of the isolates was obtained through a thermal lysis
168 protocol established by Zagui et al. (2021). A NanoDrop2000 spectrophotometer (Thermo
169 Scientific) was used to assess both the quantity and quality of gDNA. The mechanisms
170 underlying bacterial resistance to 3rd- and 4th-generation cephalosporins and
171 carbapenems have significant effects on clinical practice for patient care. Therefore, the
172 study was focused on the genes that code for the ESBL and carbapenemases by multiplex
173 Polymerase Chain Reaction (PCR), using GoTaq® G2 Hot Start Taq Polymerase kit
174 (Promega Corporation, Madison, WI, USA). The antibiotic-resistant genes (ARG) here
175 investigated were the following: a) ESBL: *bla*_{CTX-M group-1, 2, 8, 9, 25}, *bla*_{PER}, *bla*_{VEB}, *bla*_{BEL},

176 *bla*_{GES} (some variants show carbapenemase activity) and b) carbapenemases: *bla*_{KPC},
177 *bla*_{IMP}, *bla*_{NDM}, *bla*_{VIM}, and *bla*_{OXA-48}.

178 Metal tolerance genes (MTG) that encode various mechanisms against widely
179 used metals in clinical practice, or having antimicrobial potential, were investigated. As
180 above-mentioned, some metals are a promising tool as potential antimicrobial therapy
181 against specific antibiotic-resistant bacteria (Lemire et al., 2013). These genes include:
182 *silA* (silver tolerance), *pcoD* (copper tolerance), *merA* (mercury tolerance), *arsB* (arsenic
183 tolerance), *terF* (tellurium tolerance), and *czcA* (tolerance to cadmium, zinc and cobalt).
184 As well as for ARG, MTG were investigated by PCR with GoTaq® G2 Hot Start Taq
185 Polymerase kit (Promega Corporation, Madison, WI, USA).

186 Both ARG and MTG were detected using the Mastercycler® ep (Eppendorf)
187 thermocycler. The following conditions were used for gene amplification: initial DNA
188 denaturation (95 °C, for 10 min), followed by 30 cycles of DNA denaturation (95 °C, for
189 1 min), primer annealing (Table 1), and DNA extension (72 °C, for 1 min). After the 30
190 cycles, the sample underwent final DNA extension for 10 min at 72 °C, followed by
191 amplicon separation by agarose gel electrophoresis (1.5%), which was observed using an
192 AlphaImager (Alpha Innotech) photocomputer under UV light. In all the assays, positive
193 and negative controls were applied. Primers used for ARG and MTG detection are shown
194 in Table 1.

195

196 **3. Results and discussion**

197 Up to 14 isolates belonging to *Enterobacter* spp., phenotypically producers of
198 ESBL or carbapenemases, were obtained from water samples (Table 2). Seven isolates
199 were identified as *E. bugandensis*, five isolates as *E. kobei*, and two isolates as *E. cloacae*.
200 In the scientific literature, there are few studies about *E. bugandensis* species, which was

201 first identified during a septicaemia outbreak in Tanzania (Doijad et al., 2016). Recently,
202 a cohort study in France showed that all fatal septic shocks were attributed to *E.*
203 *bugandensis*, which also demonstrated its high virulence (Girlich et al., 2021). The *E.*
204 *kobei* species was initially identified in Japan at the end of the 20th century, and numerous
205 human infections have been so far documented (Kosako et al., 1996; Kim et al., 2021;
206 Manandhar et al., 2022). Both species belong to *Enterobacter cloacae* Complex (EcC),
207 which are opportunistic pathogens commonly associated with nosocomial infections and
208 capable to acquire several survivor determinants with epidemic lineages (Annavajhala et
209 al., 2019). Given their known pathogenic potential, as well as the ability of these species
210 to acquire and exchange genes, the presence of these species in aquatic samples highlights
211 the need of a microbiological control at the sampling sites, by the occurrence of bacteria
212 with epidemic potential.

213 The antibiotic susceptibility profile of *Enterobacter* spp. isolates against different
214 classes of antibiotics is depicted in Figure 1. Overall, the isolates exhibited phenotypical
215 resistance to β -lactam antibiotics, probably because these antibiotics are frequently
216 prescribed in clinical settings for the treatment of nosocomial and community infections.
217 All isolates were resistant to penicillins associated with β -lactamases inhibitors
218 (excepting *E. bugandensis* TUW66 isolate), to cefoxitin, 3rd-generation cephalosporins,
219 and aztreonam. Resistance to non- β -lactam antibiotics was variable, with the exception
220 of norfloxacin, suggesting that these antibiotics remain as viable options for antibiotic
221 therapy. This is especially applicable to chloramphenicol, for which only *E. bugandensis*
222 TUW66 showed resistance. Interestingly, *E. bugandensis* HW21 isolate from hospital
223 wastewater samples should be highlighted. It exhibited resistance to 18 out of the 19
224 tested antibiotics. Moreover, considering their phenotypic resistance pattern against
225 clinical relevance antibiotics (e.g., 3rd- and 4th-generation cephalosporins, ertapenem

226 and quinolones), the isolates from urban wastewater and surface water were also relevant.
227 The occurrence of these isolates in the environment, especially in surface water, is an
228 issue of concern due to the inappropriate use of these waters by the population living
229 close to watercourse, either for primary contact recreation, or as water catchment for
230 different purposes.

231 In a recent study conducted with different Gram-negative bacilli species from
232 hospital wastewater, Galarde-López et al. (2022) reported a broader susceptibility of *E.*
233 *bugandensis* and *E. cloacae*, as both displayed only resistance to cefoxitin. This means a
234 completely different scenario (Figure 1). It is known that the intensive use of certain
235 antibiotic classes favors resistance spread. According to the WHO Report on Surveillance
236 of Antibiotic Consumption (WHO, 2018), β -lactam antibiotics are the most consumed in
237 Brazil. This fact is in agreement with the occurrence of resistance here observed
238 especially in isolates obtained from hospital effluent samples, where the antibiotics
239 consumption is more expressive. On the other hand, non- β -lactam antibiotics are less
240 consumed in Brazil (WHO, 2018). Consequently, the susceptibility to these antibiotics
241 and their resistance mechanisms are less spread, when compared to β -lactam resistance
242 mechanisms.

243 The MAR index of the isolates ranged between 0.42 and 0.95 (Figure 2). Indexes
244 above cut-off point (0.2) could mean sources of high-risk for bacterial resistance. In all
245 the sampling points, *Enterobacter* spp. was resistant to at least eight antibiotics (MAR
246 index > 0.4), excepting the upstream sampling site, where *Enterobacter* spp. was not
247 detected. In addition, the MAR index above of cut-off point denotes that the isolates have
248 been in environments where antibiotic use is constant (Mapipa et al., 2022). On the other
249 hand, *E. bugandensis* RUW160, *E. bugandensis* TUW66 and *E. cloacae* DS81 isolates

250 from non-hospital settings also presented a MAR index above the cut-off point, which
251 draws attention to the waterborne spread of MDR bacteria.

252 The present study is focused on β -lactam resistance mechanisms encoded by *bla*
253 genes due to its relevance to clinical practice and common location on mobile genetic
254 elements usually transferable by horizontal gene transfer to other bacteria. The
255 distribution of genetic elements found in all the isolates, related to β -lactam resistance
256 and to metal tolerance, is shown in Table 3. After the assays to investigate 14 *bla* genes
257 that encode acquired carbapenemases or ESBL, *bla*_{KPC} or *bla*_{CTX-M} genes were found in
258 12 isolates. Moreover, *bla*_{KPC} was mainly detected in isolates from hospital wastewater.
259 It might explain the phenotypic resistance against β -lactam antibiotics observed in some
260 isolates, being related to the hydrolysis activity of carbapenems, extended-spectrum
261 cephalosporins, aztreonam and penicillins (Bush and Bradford, 2020). In *E. bugandensis*
262 HW6, the *bla*_{CTX-M-Gp1} gene, that encodes an ESBL enzyme, was detected, which justifies
263 β -lactam resistance. Regarding carbapenems resistance, other mechanisms might be
264 involved, including non-investigated *bla* genes, or mutations on genes coding for outer
265 membrane protein, porin (e.g. *ompK37*) (Doménech-Sánchez et al., 1999). The particular
266 occurrence of these two genes is a concern considering the genetic arrangements in which
267 *bla*_{KPC} is usually inserted in Tn4401 transposon and *bla*_{CTX-M} as cassette gene in class 1
268 integron, both with a high mobility (Cantón and Coque, 2006; Cuzon et al., 2011).

269 Because of its antibacterial activity, possibility for therapy, and lack of
270 epidemiological distribution data, metal tolerance genes were here investigated in
271 addition to the ARGs (Table 3). The most abundant metal tolerance genes were *silA* and
272 *pcoD*, which are components of silver- and copper-operons, respectively. These encode
273 efflux pumps responsible for these metals' extrusion (Hobman and Crossman, 2015;
274 Andrade et al., 2018). Silver and copper tolerance genes occurred in the same isolates,

275 confirming the CHASRI region dispersion in *Enterobacter* spp., where both tolerance
276 mechanisms are adjacent (Stahlin et al., 2016). Other metals tolerance mechanisms were
277 also found, including mercuric reductase encoded by *merA* gene, tellurium resistance
278 protein, and arsenic efflux pump encoded by *terF* and *arsB* genes, respectively. Some of
279 these metals play a role for medical purposes (i.e., topical creams, surface coating for
280 biofilm prevention, or eye care solutions, for example), and also in agricultural activities
281 and animal food supplementation (Mijnendonckx et al., 2013; Hobman and Crossman,
282 2015). Metals are widely spread in the environment. Given that bacteria are largely
283 exposed to trace elements, the development and improvement of these tolerance
284 mechanisms may mean an additional problem for the use metals as antimicrobial agents.
285 Furthermore, it suggests that silver and copper tolerance mechanisms tend to be endemic
286 in *Enterobacter* spp.

287 Other EcC species containing the described ARG and MTG have been
288 documented in the scientific literature. It includes isolates obtained from clinical samples
289 at the same hospital under investigation (Andrade et al., 2018; Galetti et al., 2022).
290 However, data on the species *E. bugandensis* and *E. kobei* are limited. The isolates studied
291 were characterized as carbapenem-resistant and ESBL-producing pathogen. Therefore,
292 they can be recognized as a critical priority by WHO, for which new antimicrobial actives
293 development and control measures are urgently required (WHO, 2017). Taking into
294 account the physicochemical properties of aquatic matrices, the detection of resistant
295 isolates to main antibiotics and co-harbored β -lactamase, and metal tolerance encoding
296 genes, imply major dangers for public and environmental health. In this scenario, a
297 number of variables can be considered: load of organic matter, cell-to-cell contact,
298 retention time (in UWWTP), and chemicals with antimicrobial potential, which can select
299 ARB and favor genetic elements exchanges (Manaia et al., 2018). The development of

300 new antibiotics, a reduction in the use and abuse of antibiotics, the implementation of
301 hospital wastewater treatment, and additional wastewater treatment technologies in
302 UWWTP, are just a few of the multidisciplinary measures to be followed in order to stop
303 the spread and fight ARB, under the One Health approach (McEwen and Collignon,
304 2018). Currently, there exist complementary technologies for the removal of chemical
305 and microbiological contaminants. Unfortunately, they may require large area or high
306 costs. Taking into account the financial constraints in developing countries, the treatment
307 of hospital and urban effluents should be tackled with efficient and affordable techniques,
308 including the hybrid technology that removes antimicrobial agents (antibiotics, metals,
309 disinfectants), microorganisms, and even microplastics (Kruglova et al., 2022; Zagui et
310 al., 2022). Anyway, the control of AMR spread is recognized as one of the greatest threats
311 to global health.

312

313 **4. Conclusions**

314 This study shows a phenotypic and genotypic characterization of antibiotic
315 resistance and metal tolerance mechanisms in *Enterobacter* spp. from water matrices.
316 Isolates co-harboring carbapenemase and ESBL genes (*bla_{KPC}* and *bla_{CTX-M}*), which are
317 known to negatively affect public health, and contribute to resistance to antibiotics
318 commonly used for treating MDR bacteria, were found. Additionally, the results of the
319 current investigation contribute to the epidemiological characterization of metal tolerance
320 genes dispersion. Specifically, a high dissemination of silver and copper tolerance genes
321 was observed. Considering the notable knowledge gaps in the scientific literature, the
322 characterization of antimicrobial resistance/tolerance of *E. bugandensis* and *E. kobei*
323 species must be a priority. The presence of these bacteria in the analyzed matrices
324 highlights the need for containment measures. The success of MDR bacteria control is

325 dependent on multidisciplinary measures, including those established by the WHO
326 Global Action Plan. Thus, for example, future actions must enhance the raise of awareness
327 through communication and education, hence optimizing the use of antimicrobials in
328 human health, but also in animal health, given the significant use in food-producing
329 animals.

330

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335

336 **Declaration of competing interest**

337 The authors declare that they have no known competing financial interests or
338 personal relationships that could have appeared to influence the work reported in this
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1 **Multidrug-resistant *Enterobacter* spp. in wastewater and surface water:**
2 **Molecular characterization of β -lactam resistance and metal tolerance**
3 **genes**

4
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27 **ABSTRACT**

28 Among the ESKAPE group pathogens, *Enterobacter* spp. is an opportunistic Gram-
29 negative bacillus, widely dispersed in the environment, that causes infections. In the
30 present study, samples of hospital wastewater, raw and treated urban wastewater, as well
31 as surface receiving water, were collected to assess the occurrence of multidrug-resistant
32 (MDR) *Enterobacter* spp. A molecular characterization of β -lactam antibiotic resistance
33 and metal tolerance genes was performed. According to identification by MALDI-TOF
34 MS, 14 isolates were obtained: 7 *E. bugandensis*, 5 *E. kobei*, and 2 *E. cloacae*. The
35 isolates showed resistance mainly to β -lactam antibiotics, including those used to treat
36 infections caused by MDR bacteria. Multiple antibiotic resistance index was calculated
37 for all isolates. It allowed verify whether sampling points showed a high risk due to
38 antibiotic resistant *Enterobacter* spp., as well as to determine if the isolates have been in
39 environments with a frequent antibiotic use. Twelve isolates showed β -lactam antibiotic
40 resistance gene, being the *bla*_{KPC} widely detected. Regarding metal tolerance, 13 isolates
41 showed at least two genes that encode metal tolerance mechanisms. Overall, metal
42 tolerance mechanisms to silver, copper, mercury, arsenic and tellurium were found. New
43 data on metal tolerance mechanisms dispersion and antibiotic-resistance characterization
44 of the *E. bugandensis* and *E. kobei* species were here provided. The occurrence of MDR
45 *Enterobacter* spp. in analyzed samples draws attention to an urgent need to put control
46 measures into practice. It also evidences waterborne spread of clinically important
47 antibiotic-resistant bacteria recognized as critical priority pathogens.

48

49 *Keywords:* ESKAPE, antibiotic resistance, carbapenemase, ESBL, wastewater, surface
50 water.

51

52 **1. Introduction**

53 According to the World Health Organization (WHO), antimicrobial resistance
54 (AMR) is recognized among the ten threats to global health by antibiotic resistance (AR).
55 It contributes not only to high mortality rates, but also to economic impacts related to
56 costs for treating infections (WHO, 2019). Only in 2019, antibiotic-resistant bacteria
57 (ARB) were associated to the death of more than 1.2 million people, with bacteria
58 belonging to ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*
59 *pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.)
60 group pathogens as the most frequent etiologic agents (Murray et al., 2022). *Enterobacter*
61 spp. are mobile Gram-negative bacilli part of gut microbiota of animals and humans. They
62 have an opportunistic behavior, which causes serious nosocomial infections highlighting
63 the potential adverse health effects in patients under mechanical ventilation in intensive
64 care units (Davin-Regli et al., 2019).

65 To date, more than 20 species belonging to the genus *Enterobacter* have been
66 identified. Among them, *E. cloacae* and *E. hormaechei* are the most frequently associated
67 with clinical infections. However, recently other species, such as *E. kobei* and *E.*
68 *bugandensis*, have drawn attention for their ability to cause infections and outbreaks
69 (Manandhar et al., 2022; Pati et al., 2018). Due to AmpC β -lactamase expression, these
70 opportunistic pathogens are intrinsically resistant to amoxicillin, ampicillin, cefoxitin and
71 to first generation cephalosporins, while in intra-hospital settings are commonly
72 associated with multidrug-resistant (MDR) phenotype. This means a resistance profile to
73 three or more antibiotics classes, as a consequence of the ability to acquire several mobile
74 genetics elements harboring resistance genes (Davin-Regli et al., 2019; Magiorakos et al.,
75 2012).

76 Carbapenems and 3rd- and 4th-generation cephalosporins are often used to treat
77 infections by MDR *Enterobacter* spp. However, the wide distribution of coding genes for
78 carbapenemases and extended-spectrum β -lactamases (ESBL) in *Enterobacterales*
79 family, including *Enterobacter* spp., compromise the therapy with these antibiotics (Bush
80 and Bradford, 2020). Considering the risk to global health by antibiotic resistance, in 2017
81 the WHO established a priority pathogen list in which new antimicrobials are needed
82 (WHO, 2017). In relation to this, metals such as silver, copper and mercury are promising
83 for bacterial infections treatment due to their antimicrobial potential. Notwithstanding,
84 some metal tolerance mechanisms might compromise their viability as antimicrobial
85 agents. Anyhow, the epidemiological distribution of these tolerance mechanisms remains
86 still unknown (Hobman and Crossman, 2015; Zagui et al., 2021).

87 MDR *Enterobacter* spp. is frequently detected in intra-hospital environments
88 (Boutarfi et al., 2019; Chen et al., 2021; Liao et al., 2022). Special attention has been paid
89 to hospital wastewater, taking into account its potential to harbor ARB and allow its
90 spread to other environments, including urban wastewater and surface water (Hocquet et
91 al., 2016; Zhu et al., 2023). A number of studies around the world have confirmed the
92 presence of ARB in different kinds of aquatic matrices (Kutilova et al., 2021; Kang et al.,
93 2022; Noman et al., 2023). In Brazil, as well as in other developing countries, hospital
94 wastewater treatment is mandatory only for those cities, which do not have urban
95 wastewater treatment plants (UWWTP) (Carraro et al., 2016). On the other hand,
96 allowing discharged of untreated hospital wastewater may favor the pathogens spread
97 through the sewage galleries, increasing the microbial load on the UWWTP. This practice
98 does not guarantee the complete removal of these microorganisms with secondary
99 treatment, as already evidenced by its presence in treated effluents (Manaia et al., 2018;
100 Kumar et al., 2020).

101 The present study was aimed at determining the occurrence of MDR *Enterobacter*
102 spp. in aquatic matrices. Considering the pathogenic potential of that organism for both,
103 humans and animals, the genotype for β -lactam antibiotic resistance and metal tolerance
104 were also investigated.

105

106 **2. Materials and methods**

107 *2.1. Study area and sample collection*

108 Ribeirão Preto city is located at the northeast of São Paulo State, Brazil. It has a
109 large population (>710,000 inhabitants) and many tertiary hospitals (IBGE, 2023). One
110 of them is a sizable university hospital, which covers an area of more than 4 million
111 inhabitants, whose untreated wastewater is disposed into a public collection system
112 according to the current legal regulation RDC 50/2002 (Brazilian Health Regulatory
113 Agency, 2002). The collected urban and hospital wastewater is carried to the UWWTP,
114 which uses activated sludge technology for treatment with no tertiary treatment.
115 Ultimately, the treated wastewater is disposed on an urban stream. In order to evaluate
116 the microbial dispersion in different aquatic matrices, water samples were collected at
117 different locations: the tertiary hospital (untreated wastewater collected at confluence
118 point of the effluents from different sectors of the hospital, such as wards, ambulatories
119 and also laboratories), the UWWTP (raw and treated wastewater), and the urban stream,
120 that receives treated wastewater by UWWTP (upstream and downstream). The upstream
121 samples were collected 350 m away from the treated effluent discharge point into the
122 river, while the downstream samples were collected 300 m away from the effluent
123 discharge point. Water samples of 1 L were collected between March and September 2022
124 and stored according to the Standards Methods for the Examination of Water and
125 Wastewater (APHA/AWWA/WEF, 2017).

126

127 *2.2. Sample processing, bacterial isolation and identification*

128 Samples were subjected to serial dilution and vacuum filtration through cellulose
129 ester membrane. Subsequently, they were put in Petri dishes containing selective
130 chromogenic medium for isolation of carbapenemase- and ESBL-producing bacteria,
131 according to the manufacturer's instructions (Probac®, Brazil) (Haller et al., 2018). After
132 the incubation period (24 h), colonies with green colors (presumptive for *Enterobacter*
133 spp.) were selected for further analysis. The isolates were grown on MacConkey agar to
134 obtain pure colonies, being stored at -80°C in Brain Heart Infusion broth supplemented
135 with glycerol (20%). The isolates were identified with Matrix-Assisted Laser
136 Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS, Bruker
137 Daltonics, Billerica, MA, USA) (Dingle and Butler-Wu, 2013).

138

139 *2.3. Antibiotic susceptibility testing*

140 The isolates were tested against 19 antibiotics -belonging to different classes- by
141 disc-diffusion method according to the Brazilian and the European Committees of
142 Antimicrobial Susceptibility Testing (BrCAST, 2022; EUCAST, 2022). Briefly, a
143 suspension of the bacterial isolates in 0.9% NaCl solution was done, similar to a turbidity
144 degree of 0.5 on the McFarland scale. After this, a cotton swab was submerged on the
145 suspension with subsequent seeding on Petri dishes containing Mueller-Hinton Agar.
146 Antibiotic discs (Oxoid) were disposed with automatic dispenser on the plates, proceeded
147 by 24 h incubation period at $35 \pm 1^\circ\text{C}$. The following antibiotics were used: amoxicillin-
148 clavulanate, ampicillin-sulbactam, piperacillin-tazobactam, cefepime, ceftazidime,
149 cefotaxime, cefoxitin, aztreonam, ertapenem, imipenem, meropenem, ciprofloxacin,
150 levofloxacin, norfloxacin, amikacin, tobramycin, gentamycin, sulfamethoxazole-

151 trimethoprim, and chloramphenicol. *Escherichia coli* ATCC 25922 strain was used as
152 quality control.

153

154 *2.4. Multiple antibiotic resistance (MAR) index*

155 The MAR index is a numerical representation of the degree to which one bacterial
156 isolate is resistant in relation to all the tested antibiotics. It is widely used to verify high-
157 risk sources for antibiotic resistance. When the index exceeds 0.2, it is considered that the
158 isolated bacterium has previously been in situations in which antibiotics were employed
159 (Krumperman, 1983).

160 MAR index was calculated according to the following equation:

$$161 \text{MAR}_{\text{index}} = \frac{a}{b}$$

162 where a represents the number of antibiotics for which a bacterial isolate exhibits
163 resistance phenotype, being b the total number of tested antibiotics ($n = 19$ antibiotics,
164 see section 2.3) (Krumperman, 1983).

165

166 *2.5. Molecular investigation of genes coding for β -lactamase and metal tolerance*

167 Genomic DNA (gDNA) of the isolates was obtained through a thermal lysis
168 protocol established by Zagui et al. (2021). A NanoDrop2000 spectrophotometer (Thermo
169 Scientific) was used to assess both the quantity and quality of gDNA. The mechanisms
170 underlying bacterial resistance to 3rd- and 4th-generation cephalosporins and
171 carbapenems have significant effects on clinical practice for patient care. Therefore, the
172 study was focused on the genes that code for the ESBL and carbapenemases by multiplex
173 Polymerase Chain Reaction (PCR), using GoTaq® G2 Hot Start Taq Polymerase kit
174 (Promega Corporation, Madison, WI, USA). The antibiotic-resistant genes (ARG) here
175 investigated were the following: a) ESBL: $bla_{\text{CTX-M group-1, 2, 8, 9, 25}}$, bla_{PER} , bla_{VEB} , bla_{BEL} ,

176 *bla*_{GES} (some variants show carbapenemase activity) and b) carbapenemases: *bla*_{KPC},
177 *bla*_{IMP}, *bla*_{NDM}, *bla*_{VIM}, and *bla*_{OXA-48}.

178 Metal tolerance genes (MTG) that encode various mechanisms against widely
179 used metals in clinical practice, or having antimicrobial potential, were investigated. As
180 above-mentioned, some metals are a promising tool as potential antimicrobial therapy
181 against specific antibiotic-resistant bacteria (Lemire et al., 2013). These genes include:
182 *silA* (silver tolerance), *pcoD* (copper tolerance), *merA* (mercury tolerance), *arsB* (arsenic
183 tolerance), *terF* (tellurium tolerance), and *czcA* (tolerance to cadmium, zinc and cobalt).
184 As well as for ARG, MTG were investigated by PCR with GoTaq® G2 Hot Start Taq
185 Polymerase kit (Promega Corporation, Madison, WI, USA).

186 Both ARG and MTG were detected using the Mastercycler® ep (Eppendorf)
187 thermocycler. The following conditions were used for gene amplification: initial DNA
188 denaturation (95 °C, for 10 min), followed by 30 cycles of DNA denaturation (95 °C, for
189 1 min), primer annealing (Table 1), and DNA extension (72 °C, for 1 min). After the 30
190 cycles, the sample underwent final DNA extension for 10 min at 72 °C, followed by
191 amplicon separation by agarose gel electrophoresis (1.5%), which was observed using an
192 AlphaImager (Alpha Innotech) photocomenter under UV light. In all the assays, positive
193 and negative controls were applied. Primers used for ARG and MTG detection are shown
194 in Table 1.

195

196 **3. Results and discussion**

197 Up to 14 isolates belonging to *Enterobacter* spp., phenotypically producers of
198 ESBL or carbapenemases, were obtained from water samples (Table 2). Seven isolates
199 were identified as *E. bugandensis*, five isolates as *E. kobei*, and two isolates as *E. cloacae*.
200 In the scientific literature, there are few studies about *E. bugandensis* species, which was

201 first identified during a septicaemia outbreak in Tanzania (Doijad et al., 2016). Recently,
202 a cohort study in France showed that all fatal septic shocks were attributed to *E.*
203 *bugandensis*, which also demonstrated its high virulence (Girlich et al., 2021). The *E.*
204 *kobei* species was initially identified in Japan at the end of the 20th century, and numerous
205 human infections have been so far documented (Kosako et al., 1996; Kim et al., 2021;
206 Manandhar et al., 2022). Both species belong to *Enterobacter cloacae* Complex (EcC),
207 which are opportunistic pathogens commonly associated with nosocomial infections and
208 capable to acquire several survivor determinants with epidemic lineages (Annavajhala et
209 al., 2019). Given their known pathogenic potential, as well as the ability of these species
210 to acquire and exchange genes, the presence of these species in aquatic samples highlights
211 the need of a microbiological control at the sampling sites, by the occurrence of bacteria
212 with epidemic potential.

213 The antibiotic susceptibility profile of *Enterobacter* spp. isolates against different
214 classes of antibiotics is depicted in Figure 1. Overall, the isolates exhibited phenotypical
215 resistance to β -lactam antibiotics, probably because these antibiotics are frequently
216 prescribed in clinical settings for the treatment of nosocomial and community infections.
217 All isolates were resistant to penicillins associated with β -lactamases inhibitors
218 (excepting *E. bugandensis* TUW66 isolate), to ceftiofuran, 3rd-generation cephalosporins,
219 and aztreonam. Resistance to non- β -lactam antibiotics was variable, with the exception
220 of norfloxacin, suggesting that these antibiotics remain as viable options for antibiotic
221 therapy. This is especially applicable to chloramphenicol, for which only *E. bugandensis*
222 TUW66 showed resistance. Interestingly, *E. bugandensis* HW21 isolate from hospital
223 wastewater samples should be highlighted. It exhibited resistance to 18 out of the 19
224 tested antibiotics. Moreover, considering their phenotypic resistance pattern against
225 clinical relevance antibiotics (e.g., 3rd- and 4th-generation cephalosporins, ertapenem

226 and quinolones), the isolates from urban wastewater and surface water were also relevant.
227 The occurrence of these isolates in the environment, especially in surface water, is an
228 issue of concern due to the inappropriate use of these waters by the population living
229 close to watercourse, either for primary contact recreation, or as water catchment for
230 different purposes.

231 In a recent study conducted with different Gram-negative bacilli species from
232 hospital wastewater, Galarde-López et al. (2022) reported a broader susceptibility of *E.*
233 *bugandensis* and *E. cloacae*, as both displayed only resistance to cefoxitin. This means a
234 completely different scenario (Figure 1). It is known that the intensive use of certain
235 antibiotic classes favors resistance spread. According to the WHO Report on Surveillance
236 of Antibiotic Consumption (WHO, 2018), β -lactam antibiotics are the most consumed in
237 Brazil. This fact is in agreement with the occurrence of resistance here observed
238 especially in isolates obtained from hospital effluent samples, where the antibiotics
239 consumption is more expressive. On the other hand, non- β -lactam antibiotics are less
240 consumed in Brazil (WHO, 2018). Consequently, the susceptibility to these antibiotics
241 and their resistance mechanisms are less spread, when compared to β -lactam resistance
242 mechanisms.

243 The MAR index of the isolates ranged between 0.42 and 0.95 (Figure 2). Indexes
244 above cut-off point (0.2) could mean sources of high-risk for bacterial resistance. In all
245 the sampling points, *Enterobacter* spp. was resistant to at least eight antibiotics (MAR
246 index > 0.4), excepting the upstream sampling site, where *Enterobacter* spp. was not
247 detected. In addition, the MAR index above of cut-off point denotes that the isolates have
248 been in environments where antibiotic use is constant (Mapipa et al., 2022). On the other
249 hand, *E. bugandensis* RUW160, *E. bugandensis* TUW66 and *E. cloacae* DS81 isolates

250 from non-hospital settings also presented a MAR index above the cut-off point, which
251 draws attention to the waterborne spread of MDR bacteria.

252 The present study is focused on β -lactam resistance mechanisms encoded by *bla*
253 genes due to its relevance to clinical practice and common location on mobile genetic
254 elements usually transferable by horizontal gene transfer to other bacteria. The
255 distribution of genetic elements found in all the isolates, related to β -lactam resistance
256 and to metal tolerance, is shown in Table 3. After the assays to investigate 14 *bla* genes
257 that encode acquired carbapenemases or ESBL, *bla*_{KPC} or *bla*_{CTX-M} genes were found in
258 12 isolates. Moreover, *bla*_{KPC} was mainly detected in isolates from hospital wastewater.
259 It might explain the phenotypic resistance against β -lactam antibiotics observed in some
260 isolates, being related to the hydrolysis activity of carbapenems, extended-spectrum
261 cephalosporins, aztreonam and penicillins (Bush and Bradford, 2020). In *E. bugandensis*
262 HW6, the *bla*_{CTX-M-Gp1} gene, that encodes an ESBL enzyme, was detected, which justifies
263 β -lactam resistance. Regarding carbapenems resistance, other mechanisms might be
264 involved, including non-investigated *bla* genes, or mutations on genes coding for outer
265 membrane protein, porin (e.g. *ompK37*) (Doménech-Sánchez et al., 1999). The particular
266 occurrence of these two genes is a concern considering the genetic arrangements in which
267 *bla*_{KPC} is usually inserted in Tn4401 transposon and *bla*_{CTX-M} as cassette gene in class 1
268 integron, both with a high mobility (Cantón and Coque, 2006; Cuzon et al., 2011).

269 Because of its antibacterial activity, possibility for therapy, and lack of
270 epidemiological distribution data, metal tolerance genes were here investigated in
271 addition to the ARGs (Table 3). The most abundant metal tolerance genes were *silA* and
272 *pcoD*, which are components of silver- and copper-operons, respectively. These encode
273 efflux pumps responsible for these metals' extrusion (Hobman and Crossman, 2015;
274 Andrade et al., 2018). Silver and copper tolerance genes occurred in the same isolates,

275 confirming the CHASRI region dispersion in *Enterobacter* spp., where both tolerance
276 mechanisms are adjacent (Staeclin et al., 2016). Other metals tolerance mechanisms were
277 also found, including mercuric reductase encoded by *merA* gene, tellurium resistance
278 protein, and arsenic efflux pump encoded by *terF* and *arsB* genes, respectively. Some of
279 these metals play a role for medical purposes (i.e., topical creams, surface coating for
280 biofilm prevention, or eye care solutions, for example), and also in agricultural activities
281 and animal food supplementation (Mijnendonckx et al., 2013; Hobman and Crossman,
282 2015). Metals are widely spread in the environment. Given that bacteria are largely
283 exposed to trace elements, the development and improvement of these tolerance
284 mechanisms may mean an additional problem for the use metals as antimicrobial agents.
285 Furthermore, it suggests that silver and copper tolerance mechanisms tend to be endemic
286 in *Enterobacter* spp.

287 Other EcC species containing the described ARG and MTG have been
288 documented in the scientific literature. It includes isolates obtained from clinical samples
289 at the same hospital under investigation (Andrade et al., 2018; Galetti et al., 2022).
290 However, data on the species *E. bugandensis* and *E. kobei* are limited. The isolates studied
291 were characterized as carbapenem-resistant and ESBL-producing pathogen. Therefore,
292 they can be recognized as a critical priority by WHO, for which new antimicrobial actives
293 development and control measures are urgently required (WHO, 2017). Taking into
294 account the physicochemical properties of aquatic matrices, the detection of resistant
295 isolates to main antibiotics and co-harbored β -lactamase, and metal tolerance encoding
296 genes, imply major dangers for public and environmental health. In this scenario, a
297 number of variables can be considered: load of organic matter, cell-to-cell contact,
298 retention time (in UWWTP), and chemicals with antimicrobial potential, which can select
299 ARB and favor genetic elements exchanges (Manaia et al., 2018). The development of

300 new antibiotics, a reduction in the use and abuse of antibiotics, the implementation of
301 hospital wastewater treatment, and additional wastewater treatment technologies in
302 UWWTP, are just a few of the multidisciplinary measures to be followed in order to stop
303 the spread and fight ARB, under the One Health approach (McEwen and Collignon,
304 2018). Currently, there exist complementary technologies for the removal of chemical
305 and microbiological contaminants. Unfortunately, they may require large area or high
306 costs. Taking into account the financial constraints in developing countries, the treatment
307 of hospital and urban effluents should be tackled with efficient and affordable techniques,
308 including the hybrid technology that removes antimicrobial agents (antibiotics, metals,
309 disinfectants), microorganisms, and even microplastics (Kruglova et al., 2022; Zagui et
310 al., 2022). Anyway, the control of AMR spread is recognized as one of the greatest threats
311 to global health.

312

313 **4. Conclusions**

314 This study shows a phenotypic and genotypic characterization of antibiotic
315 resistance and metal tolerance mechanisms in *Enterobacter* spp. from water matrices.
316 Isolates co-harboring carbapenemase and ESBL genes (*bla_{KPC}* and *bla_{CTX-M}*), which are
317 known to negatively affect public health, and contribute to resistance to antibiotics
318 commonly used for treating MDR bacteria, were found. Additionally, the results of the
319 current investigation contribute to the epidemiological characterization of metal tolerance
320 genes dispersion. Specifically, a high dissemination of silver and copper tolerance genes
321 was observed. Considering the notable knowledge gaps in the scientific literature, the
322 characterization of antimicrobial resistance/tolerance of *E. bugandensis* and *E. kobei*
323 species must be a priority. The presence of these bacteria in the analyzed matrices
324 highlights the need for containment measures. The success of MDR bacteria control is

325 dependent on multidisciplinary measures, including those established by the WHO
326 Global Action Plan. Thus, for example, future actions must enhance the raise of awareness
327 through communication and education, hence optimizing the use of antimicrobials in
328 human health, but also in animal health, given the significant use in food-producing
329 animals.

330

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335

336 **Declaration of competing interest**

337 The authors declare that they have no known competing financial interests or
338 personal relationships that could have appeared to influence the work reported in this
339 paper.

340

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Table 1. Primers used for detection of genes coding for β -lactamases and metal tolerance mechanisms, with respective amplicon size and annealing temperature.

Gene	Primers*	Nucleotides sequences (5' – 3')	Amplicon size (pb)	Annealing T (°C)	Reference
<i>bla</i> _{NDM}	<i>bla</i> _{NDM} f <i>bla</i> _{NDM} r	ACT TGG CCT TGC TGT CCT T CAT TAG CCG CTG CAT TGA T	603	57	Bogaerts et al. (2013)
<i>bla</i> _{VIM}	<i>bla</i> _{VIM} f <i>bla</i> _{VIM} r	TGT CCG TGA TGG TGA TGA GT ATT CAG CCA GAT CGG CAT C	437	57	Bogaerts et al. (2013)
<i>bla</i> _{IMP}	<i>bla</i> _{IMP} f <i>bla</i> _{IMP} r	ACA YGG YTT RGT DGT KCT TG GGT TTA AYA AAR CAA CCA CC	387	57	Bogaerts et al. (2013)
<i>bla</i> _{KPC}	<i>bla</i> _{KPC} f <i>bla</i> _{KPC} r	TCG CCG TCT AGT TCT GCT GTC TTG ACA GCT CCG CCA CCG TCA T	353	57	Bogaerts et al. (2013)
<i>bla</i> _{OXA}	<i>bla</i> _{OXA-48} f <i>bla</i> _{OXA-48} r	ATG CGT GTA TTA GCC TTA TCG CAT CCT TAA CCA CGC CCA AAT C	265	57	Bogaerts et al. (2013)
<i>bla</i> _{CTX-M}	<i>bla</i> _{CTX-M-1} f <i>bla</i> _{CTX-M-1} r	AAA AAT CAC TGC GCC AGT TC AGC TTA TTC ATC GCC ACG TT	415	52	Woodford et al. (2006)
	<i>bla</i> _{CTX-M-2} f <i>bla</i> _{CTX-M-2} r	CGA CGC TAC CCC TGC TAT T CCA GCG TCA GAT TTT TCA GG	552	52	Woodford et al. (2006)
	<i>bla</i> _{CTX-M-8} f <i>bla</i> _{CTX-M-8} r	TCG CGT TAA GCG GAT GAT GC AAC CCA CGA TGT GGG TAG C	666	52	Woodford et al. (2006)
	<i>bla</i> _{CTX-M-9} f <i>bla</i> _{CTX-M-9} r	CAA AGA GAG TGC AAC GGA TG ATT GGA AAG CGT TCA TCA CC	205	52	Woodford et al. (2006)
	<i>bla</i> _{CTX-M-25} f <i>bla</i> _{CTX-M-25} r	GCA CGA TGA CAT TCG GG AAC CCA CGA TGT GGG TAG C	327	52	Woodford et al. (2006)
<i>bla</i> _{VEB}	<i>bla</i> _{VEB} f <i>bla</i> _{VEB} r	CGA CTT CCA TTT CCC GAT GC TGT TGG GGT TGC CCA ATT TT	376	57	Bogaerts et al. (2013)
<i>bla</i> _{PER}	<i>bla</i> _{PER} f <i>bla</i> _{PER} r	AGT GTG GGG GCC TGA CGA T GCA ACC TGC GCA ATR ATA GCT T	725	57	Bogaerts et al. (2013)
<i>bla</i> _{GES}	<i>bla</i> _{GES} f <i>bla</i> _{GES} r	CTG GCA GGG ATC GCT CAC TC TTC CGA TCA GCC ACC TCT CA	600	57	Bogaerts et al. (2013)
<i>bla</i> _{BEL}	<i>bla</i> _{BEL} f <i>bla</i> _{BEL} r	CGA CAA TGC CGC AGC TAA CC CAG AAG CAA TTA ATA ACG CCC	448	57	Bogaerts et al. (2013)
<i>silA</i>	<i>silA</i> f <i>silA</i> r	GCA AGA CCG GTA AAG CAG AG CCT GCC AGT ACA GGA ACC AT	936	59	Mourão et al. (2015)
<i>pcoD</i>	<i>pcoD</i> f <i>pcoD</i> r	CTG GCC ACA CTT GCC TGG GG CAC GCT ACG GCG CCC AGA AT	500	55	Mourão et al. (2015)
<i>merA</i>	<i>merA</i> f <i>merA</i> r	ACC ATC GGC GGC ACC TGC GT ACC ATC GTC AGG TAG GGG AAC	1238	67	Mourão et al. (2015)
<i>arsB</i>	<i>arsB</i> f <i>arsB</i> r	AGT GAA AGA CAG ACG AAG CG GGC AGA TAG TGT GGA ATG CG	1136	60	Mourão et al. (2015)
<i>czcA</i>	<i>czcA</i> f <i>czcA</i> r	GTT CAC CTT GCT CTT CGC CAT GTT ACA GGT TGC GGA TGA AGG AGA TCA	206	63	Bouskill et al. (2007)
<i>terF</i>	<i>terF</i> f <i>terF</i> r	ATG CAG GCT CAA GGA ATC GC TTC ATC GAT CCA CGG TCT G	990	63	Mourão et al. (2015)

*For degenerate primers: D = A, G, or T; R = A or G; Y = C or T; K = G or T

Table 2. Distribution and identification of *Enterobacter* spp. isolates from water matrices.

Sampling site	Species / ID
Hospital wastewater	<i>E. bugandensis</i> HW6
	<i>E. bugandensis</i> HW16
	<i>E. bugandensis</i> HW21
	<i>E. kobei</i> HW30
	<i>E. bugandensis</i> HW31
	<i>E. kobei</i> HW37
	<i>E. kobei</i> HW102
	<i>E. kobei</i> HW126
	<i>E. cloacae</i> HW127
	<i>E. bugandensis</i> HW128
<i>E. kobei</i> HW130	
Raw urban wastewater	<i>E. bugandensis</i> RUW160
Treated urban wastewater	<i>E. bugandensis</i> TUW66
Downstream	<i>E. cloacae</i> DS81

Table 3. β -lactam antibiotic resistance genes and metal tolerance genes detected in *Enterobacter* isolated from water matrices.

Isolate	β-lactam antibiotic resistance genes	Metal tolerance genes
<i>E. bugandensis</i> HW6	<i>bla</i> _{CTX-M-Gp1}	<i>silA</i> , <i>pcoD</i>
<i>E. bugandensis</i> HW16	<i>bla</i> _{KPC}	<i>arsB</i> , <i>terF</i>
<i>E. bugandensis</i> HW21	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i> , <i>merA</i> , <i>terF</i>
<i>E. kobei</i> HW30	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i>
<i>E. bugandensis</i> HW31	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i> , <i>merA</i> , <i>terF</i>
<i>E. kobei</i> HW37	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i>
<i>E. kobei</i> HW102	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i> , <i>terF</i>
<i>E. kobei</i> HW126	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i>
<i>E. cloacae</i> HW127	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i> , <i>merA</i>
<i>E. bugandensis</i> HW128	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i> , <i>merA</i>
<i>E. kobei</i> HW130	<i>bla</i> _{KPC}	<i>silA</i> , <i>pcoD</i> , <i>merA</i>
<i>E. bugandensis</i> RUW160	<i>bla</i> _{CTX-M-Gp1}	<i>silA</i> , <i>pcoD</i>
<i>E. bugandensis</i> TUW66	-	<i>silA</i> , <i>pcoD</i> , <i>merA</i>
<i>E. cloacae</i> DS81	-	-

Figure 1. Antibiotic susceptibility profile of *Enterobacter* spp. isolates from water matrices. Red circles represent resistant phenotype, while yellow circles and green circles represent intermediate and sensible phenotypes, respectively. Overall, widespread resistance to beta-lactam antibiotics was found, which might be attributed to their extensive consumption, particularly in hospital settings. Resistance to clinically relevant antibiotics, such as carbapenems, was more common among hospital isolates, highlighting differences in resistance profiles between sampling locations. *AMC*, amoxicillin-clavulanate. *ASB*, ampicillin-sulbactam. *TZP*, piperacillin-tazobactam. *FOX*, ceftoxitin. *CAZ*, ceftazidime. *CTX*, cefotaxime. *FEP*, cefepime. *ATM*, aztreonam. *ETP*, ertapenem. *IPM*, imipenem. *MEM*, meropenem. *AMK*, amikacin. *CN*, gentamycin. *TOB*, tobramycin. *CIP*, ciprofloxacin. *LEV*, levofloxacin. *NOR*, norfloxacin. *STX*, sulfamethoxazole-trimethoprim. *C*, chloramphenicol.

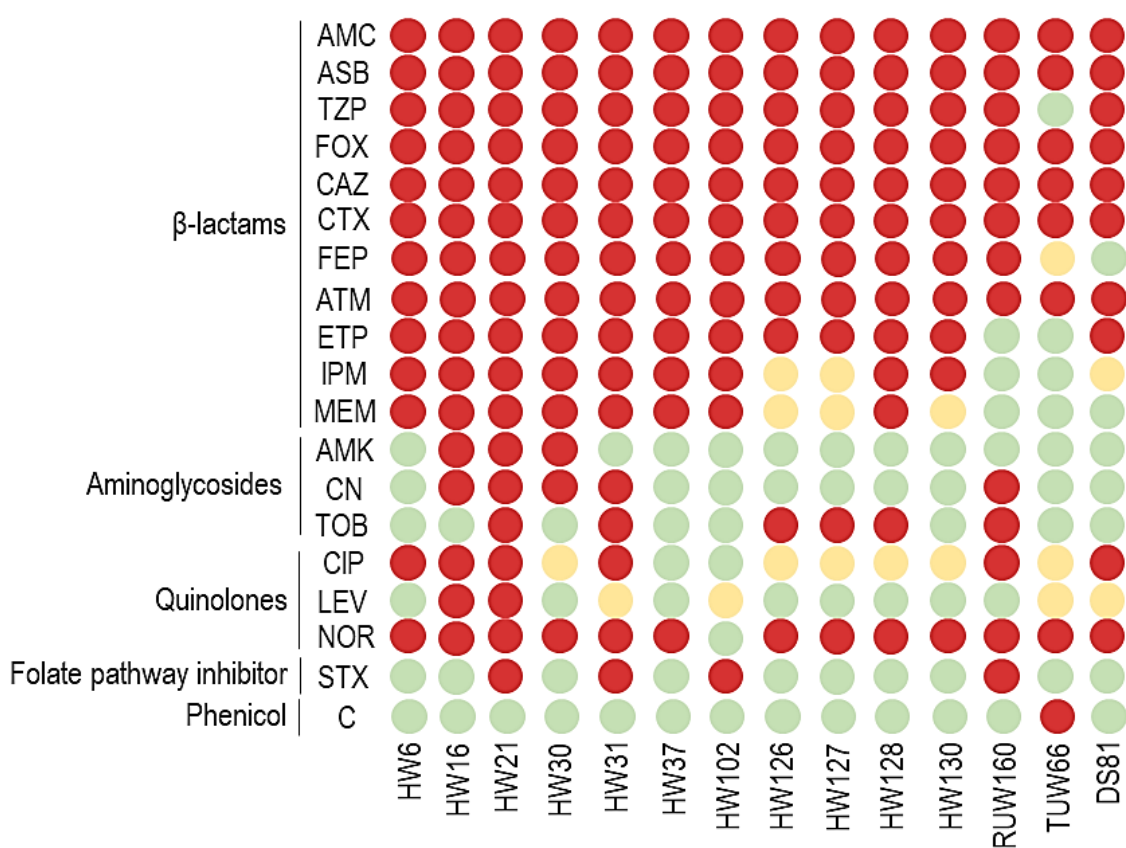


Figure 2. Multiple antibiotic resistance index (MAR Index) of *Enterobacter* spp. obtained from hospital wastewater, urban wastewater and surface water.

