

1 Molecular epidemiology of carbapenem-resistant *Enterobacter cloacae* complex infections  
2 uncovers high frequency of non-carbapenemase-producers in five tertiary care hospitals  
3 from Colombia

4

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12 Running Head: Molecular epidemiology of carbapenem-resistant *E. cloacae*

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16

17 **Abstract.**

18 **Background.** Infections caused by carbapenem-resistant *Enterobacter cloacae* (CR-Ecl)  
19 have been increasingly reported in the clinical setting; here we describe the clinical and  
20 molecular characteristics of CR-Ecl infections in a KPC endemic region.

21 **Methods.** A cross-sectional study was conducted in five tertiary-care hospitals in Medellín-  
22 Colombia. All patients infected by CR-Ecl from June-2012 to June-2014 were included.  
23 Sociodemographics and clinical information was retrieved from medical records.  
24 Antimicrobial susceptibility testing, phenotypic and molecular carbapenemase detection  
25 were performed. Analysis of *hsp60* and PFGE was done in a subset of isolates.

26 **Results.** Of 109 patients enrolled, 60.55% (66/109) were infected with non-carbapenemase-  
27 producing-Ecl (non-CP-Ecl). CP-Ecl patients were frequently hospitalized in the ICU  
28 (37.21% vs 12.12%) and had exposure to carbapenems (39.53% vs 15.15%) compared to  
29 non-CP-Ecl infected patients. All-cause 30-day mortality was higher in CP-Ecl than non-  
30 CP-Ecl infected patients (27.91% vs 19.70%). CP-Ecl harbored KPC-2 (83.72%) and KPC-  
31 3 (6.97%). Analysis of *hsp60* showed that CP-Ecl belonged primarily to cluster-VI of  
32 *Enterobacter xiangfangensis* (12/34) and cluster-XI (12/34) corresponding to *E. cloacae*  
33 subsp. *cloacae*. Non-CP-Ecl isolates belonged to cluster-VII/VIII (45/54), of *E. hormaechi*  
34 subsp. *steigerwaltii*. PFGE revealed isolates in cluster VII/VIII and XI were closely related  
35 within their own clusters.

36 **Conclusions:** The results revealed a high frequency of non-CP-Ecl among the CR-Ecl  
37 infections in a KPC endemic region, displaying distinct clinical and molecular  
38 characteristics in comparison to CP-Ecl. The study highlights a significant contribution of  
39 non-CP-Ecl to the prevalence of CR-Ecl. Infection control measures to curtail

40 dissemination of CR-Ecl should not only focus on CP-Ecl but should also include non-CP-  
41 Ecl.

42

43 **Keywords:** cross-sectional study, carbapenem-resistant, *Enterobacter cloacae* complex.

44

#### 45 **Introduction.**

46 *Enterobacter* spp. have become a significant pathogen in clinical settings in the last decades

47 (1). In fact, *Enterobacter cloacae* is among the top-five bacteria causing intra-abdominal

48 infections in hospital and community settings in all regions of the world (2). Data from the

49 National Nosocomial Infections Surveillance (NNIS) System from 1986–2003 in United

50 States showed *Enterobacter* was a frequent cause of pneumonia (10.0%), bloodstream

51 (4.4%), surgical site (9.0%) and urinary tract infections (UTI) (6.9%) among Gram-

52 negative bacilli (3) and it was mainly associated with infections occurring in intensive care

53 units (ICU) (4). Similarly, in Latin America, *Enterobacter* spp. is also among the top-five

54 Gram-negative pathogens causing bloodstream infections (4.5%), pneumonia (5.1%) and

55 skin and soft tissue infections (6.8%) (5).

56

57 *Enterobacter* spp. is increasingly associated with multi-drug resistance, including the

58 resistance to the “last-resort” carbapenems. Wilson *et al.* (6) recently described two

59 epidemics of carbapenem-resistant bacteria in United States. The first and most notorious

60 started in 2000 and it was caused by the expansion of KPC-*K. pneumoniae* from the east to

61 the pacific coast of the country, although resistance rates are decreasing in recent years.

62 The second is the unfolding epidemic caused by carbapenem-resistant *E. cloacae* (CR-Ecl)

63 extending from the east to the southwest and pacific coast of United States; in contrast to *K.*

64 *pneumoniae*, rates of carbapenem resistance appear to be growing in *E. cloacae* in recent  
65 years. Additionally, multiple outbreaks of CR-Ecl harboring KPC (7,8), VIM (9), IMP (10)  
66 and OXA-48 (11) carbapenemases have been reported globally, highlighting the key role of  
67 *Enterobacter* in dissemination of carbapenem resistance.

68  
69 In Colombia, *E. cloacae* is reported to be one of the most commonly isolated pathogens in  
70 both ICU and non-ICU wards (12). More alarmingly, the national surveillance data also  
71 showed that carbapenem-resistance rates in *E. cloacae* (10-16%) are similar to *K.*  
72 *pneumoniae* (14-15%) (12). However, KPC carbapenemases were only detected in 66% of  
73 *E. cloacae* isolates from the national program on antimicrobial resistance during 2012 to  
74 2015, suggesting additional mechanisms mediating carbapenem-resistance in CR-Ecl (13).  
75 Despite the clinical importance of this pathogen, only a few studies have focused on the  
76 characterization of infections caused by CR-Ecl. The aim of this study is to describe the  
77 clinical and microbiological characteristics of infections caused by CR-Ecl in a KPC  
78 endemic region.

79

## 80 **Materials and Methods.**

81

82 **Study design and setting.** A cross-sectional study was conducted in the city of Medellin,  
83 the second largest city in Colombia with 2.5 million inhabitants. The study was conducted  
84 in five tertiary care hospitals capturing both adult and pediatric populations. Hospital A  
85 (700-beds) and Hospital C (754-beds) are large university hospitals, Hospital B (286-beds)  
86 and Hospital D (300-beds) are medium size tertiary care centers and Hospital E (140-beds)  
87 is a small hospital specialized in cardiovascular diseases.

88

89 **Participants.** All patients infected with carbapenem-resistant *Enterobacter cloacae*  
90 complex isolates in the five tertiary care hospitals from June 2012 to June 2014 were  
91 enrolled. Patients from any age, service and type of infection were included in the study at  
92 the time of the first CR-EcI infection. Specialists in infectious diseases established the  
93 infection/colonization status of the patients using previous standardized definitions (14).  
94 The study protocol was approved by the Committee of Bioethics for Human Research at the  
95 Universidad de Antioquia (CBE-SIU) (approval no. 11-35-415) and by the Committee of  
96 Ethics at each of the participant institutions.

97

98 **Clinical information.** Information retrieved from the medical records included  
99 sociodemographics (age and sex) and the following clinical variables: time at risk (defined  
100 as the number of days from admission to the date of sampling), transfer from another  
101 facility, ICU stay, use of invasive devices at the time of sampling or 48 hours before  
102 sampling, previous healthcare exposures (surgery in the previous year, prior ICU stay and  
103 antibiotic use in the last six months), dialysis, neutropenia, immunosuppressive conditions,  
104 comorbidities (trauma, cancer, diabetes mellitus, cystic fibrosis, neurological disease,  
105 cardiovascular disease, lung disease, burns, transplant, chronic obstructive disease), health  
106 care associated infection, mixed infection, empirical and targeted treatment, discharge  
107 (death, clinical improvement, cure). All clinical information was retrieved from the medical  
108 records and included in a standardized formulary by specialists in infectious diseases at  
109 each institution.

110 **Microbial identification and antimicrobial susceptibility testing.** Identification of  
111 isolates and antimicrobial susceptibility testing was carried out by the Vitek® 2 automated

112 system (bioMérieux, Marcy l'Etoile, France). Antibiotics tested included ceftriaxone,  
113 ceftazidime, cefepime, ertapenem, imipenem, meropenem, ciprofloxacin, gentamicin,  
114 amikacin, tigecycline and colistin. Resistant, intermediate or susceptible categories were  
115 defined following CLSI guidelines (15). Isolates were considered resistant to carbapenems  
116 if at least one of the carbapenems was non-susceptible (imipenem, meropenem or  
117 doripenem MIC  $\geq$  2  $\mu$ g/mL or ertapenem MIC  $\geq$  1  $\mu$ g/mL) (15).

118

119 **Phenotypic and molecular detection of  $\beta$ -lactamases.** The three-dimensional test, which  
120 uses a mechanical lysate of the tested isolate to increase the sensitivity of carbapenemase  
121 detection (16), was performed in all CR-Ecl isolates. In addition, modified Hodge Test  
122 (MHT) (15) was conducted in a subset of 65 isolates. Molecular detection of *bla*<sub>KPC</sub>  
123 variants were done using a molecular beacon-based real-time PCR assay (17). Isoforms of  
124 Tn4401 element were evaluated by PCR (18). Detection of additional carbapenemase genes  
125 *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub> and *bla*<sub>OXA-48</sub> were done by conventional multiplex PCR (19). In  
126 addition, extended spectrum  $\beta$ -lactamases (ESBLs) genes *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-</sub>  
127 <sub>8</sub>, *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-25</sub>, *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> were evaluated using PCR and Sanger  
128 sequencing (20), and acquired AmpC genes *bla*<sub>ACT/MIR</sub>, *bla*<sub>CMY-1/MOX</sub>, *bla*<sub>CMY-2/LAT</sub>, *bla*<sub>FOX</sub>,  
129 *bla*<sub>DHA</sub> and *bla*<sub>ACC</sub> were assessed using PCR (20).

130

131 **Sequence analysis of *ompF*.** To investigate additional mechanisms related to carbapenem  
132 resistance in *E. cloacae* isolates, the full-length sequences of *ompF* were analyzed in a  
133 subset of 91 isolates. Sequences were compared to the reference strain *E. cloacae* NCTC

134 13405 (KT780421). All sequences were translated and aligned using MUSCLE accessory  
135 application in Geneious® 8.1.9 (21).

136

137 ***hsp60* phylogenetic analysis.** Sequences of *hsp60* were obtained from 91 CR-Ecl isolates  
138 using primers and conditions previously reported (22). Isolates were classified according to  
139 the Hoffman and Roggenkamp scheme (22), by comparison with reference strains of *E.*  
140 *cloacae* complex retrieved from GenBank. Sequences sets were aligned using MUSCLE  
141 accessory application in Geneious® 8.1.9 (21). Bayesian phylogenetic analysis was  
142 performed using Markov chain Monte Carlo (MCMC) sampling implemented in MrBayes  
143 3.2.6 (23), under a TPM3+G model selected according to the Bayesian Information Criteria  
144 in jmodeltest2 (24). The MCMC search was run for 3 x 10<sup>6</sup> generations with trees sampled  
145 every 500<sup>th</sup> generations and burn-in length of 200.000. Parameters estimates were assessed  
146 in Tracer v1.6 (available at <http://tree.bio.ed.ac.uk/software/tracer/>). *hsp60* sequence of *E.*  
147 *aerogenes* NBRC13534 (AB375469) was used as an outgroup.

148

149 **Strain genotyping.** Genotyping of isolates was performed by pulsed-field gel  
150 electrophoresis (PFGE) on 68 randomly selected isolates including isolates from each  
151 hospital. PFGE conditions were described previously (25). Briefly, DNA was digested with  
152 20U of *XbaI* restriction endonuclease at 37°C for two hours. PFGE conditions were initial  
153 switch time 2.2 sec, final switch time 63.8 sec, angle 120° and 6 v/cm volts. PFGE was run  
154 for 24 hours. Analysis of relatedness among *E. cloacae* complex isolates was performed on  
155 BioNumerics software version 6.0 (Applied Maths, Sint-Martens-Latem, Belgium) using  
156 the Dice coefficient and a cutoff value of 80 or higher for genetic relatedness. For  
157 dendrogram generation the unweighted-pair group method analysis with average linkages

158 (UPGMA) was used. DNA fragment patterns were normalized using a bacteriophage  
159 lambda ladder PFGE marker (New England BioLabs, United Kingdom) with a 1% position  
160 tolerance for further analysis. Six isolates from current study were characterized by  
161 WGS analysis in a previous report (26). Isolates sequenced were: EL012 (44541,  
162 accession no: NZ\_JZXU01000000), EL036 (44565, NZ\_JZXT01000000), EH005 (44517,  
163 NZ\_JZXX01000000), EH012 (44524, NZ\_JZXW01000000), EH015 (44527,  
164 NZ\_JZXV01000000) and EP004 (44589, NZ\_JZXS01000000).

165

166 **Accession numbers.** All of the *hsp60* sequences determined in this study are  
167 available at NCBI under accession numbers EL044 (MH614175), EH006 (MH614176),  
168 EH019 (MH614177), EP010 (MH614178), EL033 (MH614179), EP007 (MH614180),  
169 EH010 (MH614181), ER005 (MH614182), EH011 (MH614183), EL018 (MH614184),  
170 EP008 (MH614185), EP005 (MH614186), EC001 (MH614187), EH015 (MH614188),  
171 ER002 (MH614189), EL036 (MH614190), EL021 (MH614191), EH004 (MH614194),  
172 EC003 (MH614195), EH005 (MH614197).

173

174 **Statistical analysis.** To describe patient's characteristics, absolute and relative frequencies  
175 were used for qualitative variables, and median and interquartile range for quantitative  
176 variables with non-normal distribution. All statistical analyses were performed in  
177 STATA/IC 15.1.

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181



182 **Results.**

183 **Description of patients infected with carbapenem-resistant *E. cloacae* complex.**

184 A total 109 patients were infected with CR-Ecl during the study period. The most frequent  
185 infections in the study population were surgical site infections (n=25, 22.94%), followed by  
186 intra-abdominal (n=18, 16.51%), and urinary tract infections (UTI) (n=18, 16.51%).  
187 Overall all-cause in-hospital mortality was 24.77% (n=27) and all-cause 30-day mortality  
188 was 22.94% (n=25) (Table 1). The majority of patients were older (median age 64 years,  
189 IQR 49 – 74) and from male (n=73, 66.97%). Almost all infections in the study population  
190 were healthcare-associated (n=108, 99.08%). CR-Ecl frequently infected patients with  
191 comorbidities (92.66%) and with at least one medical device at the time of sampling or 48  
192 hours before (n=70, 64.22%), mainly urinary catheters (n=50, 45.87%). Most CR-Ecl  
193 infected patients had previous antibiotics use in the last six months (n=90, 84.11%), mostly  
194 piperacillin/tazobactam (n=51, 46.79%), fluoroquinolones (n=29, 26.61%) and  
195 carbapenems (n=17, 24.77%). Patients also have history of hospitalization in the last six  
196 months (n=82, 75.23%) and surgery in the last year (n=76, 69.72%). Additional patient's  
197 characteristics are presented in Table 1.

198 Table 1. Clinical characteristics of patients infected with carbapenem-resistant *Enterobacter cloacae* complex according KPC  
 199 detection.

200

<b>Patient characteristics</b>	<b>CP-Ecl n=43</b>	<b>Non-CP-Ecl n=66</b>	<b>Total patients n=109</b>
<b>Sociodemographics</b>			
Age in years, median (IQR)	64 (50 - 74)	63.5 (47 - 77)	64 (49 - 74)
Male sex	31 (72.09)	42 (63.64)	73 (66.97)
<b>Clinical characteristics</b>			
Transfer from another facility	13 (30.23)	16 (24.24)	29 (26.61)
Time at risk	11 (1 - 29)	6 (2 - 16)	7 (2 - 20)
ICU stay	16 (37.21)	8 (12.12)	24 (22.02)
<b>Medical devices<sup>a</sup></b>			
Urinary catheter	23 (53.49)	27 (40.91)	50 (45.87)
Vascular dialysis catheter	5 (11.63)	1 (1.52)	5 (5.50)

Parenteral Nutrition	4 (9.30)	1 (1.52)	5 (4.59)
Mechanical Ventilation	13 (30.23)	5 (7.58)	18 (16.51)
Enteral Nutrition	13 (30.23)	12 (18.18)	25 (22.94)
Central venous catheter	18 (41.86)	14 (21.21)	32 (29.36)
<b>Medical history</b>			
Previous Surgery <sup>b</sup>	28 (65.12)	48 (72.73)	76 (69.72)
Previous Hospitalization <sup>c</sup>	32 (74.42)	50 (75.76)	82 (75.23)
Previous ICU stay <sup>c</sup>	14 (32.56)	16 (24.24)	30 (27.52)
Dialysis	9 (20.93)	3 (4.55)	12 (11.01)
Immunosuppressive therapy	6 (13.95)	5 (7.58)	11 (10.09)
<b>Previous use of antibiotics<sup>c</sup></b>			
Penicillin	2 (4.65)	4 (6.06)	6 (5.50)
Carbapenems	17 (39.53)	10 (15.15)	17 (24.77)
Fluoroquinolones	12 (27.91)	17 (25.76)	29 (26.61)
Cefepime	6 (13.95)	3 (4.55)	9 (8.26)

Piperacillin/Tazobactam	23 (53.49)	28 (42.42)	51 (46.79)
Aminoglycosides	8 (18.60)	4 (6.06)	12 (11.01)
Tigecycline	2 (4.65)	1 (1.52)	3 (2.75)
<b>Comorbidities</b>	43 (100)	58 (87.88)	101 (92.66)
Trauma	5 (11.63)	15 (22.73)	20 (18.35)
Cancer	9 (20.93)	20 (30.30)	29 (26.61)
Chronic obstructive pulmonary disease	7 (16.28)	6 (9.09)	13 (11.93)
Diabetes mellitus	10 (23.26)	14 (21.21)	24 (22.02)
<b>Type of infection</b>			
Surgical site infection	7 (16.28)	18 (27.27)	25 (22.94)
Intra-abdominal infection	7 (16.28)	11 (16.67)	18 (16.51)
Urinary tract infection (UTI)	5 (11.63)	13 (19.70)	18 (16.51)
Bloodstream infection	7 (16.28)	9 (13.64)	16 (14.68)
Catheter related UTI	4 (9.30)	4 (6.06)	8 (7.34)
Ventilator associated pneumonia	2 (4.65)	2 (3.03)	4 (3.67)

Catheter related bloodstream infection	2 (4.65)	0	2 (1.83)
Pneumonia	2 (4.65)	0	2 (1.83)
<b>Empirical therapy</b>	37 (86.05)	55 (83.33)	92 (84.40)
Piperacillin/tazobactam	17 (39.53)	24 (36.36)	41 (37.61)
Carbapenems	15 (34.88)	13 (19.70)	28 (25.69)
Fourth generation cephalosporins	1 (2.33)	5 (7.58)	6 (5.50)
Aminoglycosides	4 (9.30)	5 (7.58)	9 (8.26)
Fluoroquinolones	3 (6.98)	5 (7.58)	8 (7.34)
Glycylcyclines	3 (6.98)	2 (3.03)	5 (4.59)
<b>Targeted treatment</b>	33 (76.74)	53 (80.30)	86 (78.90)
Piperacillin/tazobactam	3 (6.98)	2 (3.03)	5 (4.59)
Carbapenems	7 (16.28)	32 (48.48)	39 (35.78)
Fourth generation cephalosporins	2 (4.65)	6 (9.09)	8 (7.34)
Aminoglycosides	10 (23.26)	11 (16.67)	21 (19.27)
Fluoroquinolones	6 (13.95)	11 (16.67)	17 (15.60)

Glicilcyclines	13 (30.23)	8 (12.12)	21 (19.27)
Colistin	14 (32.56)	5 (7.58)	19 (17.43)
<b>In-hospital mortality</b>	13 (30.23)	14 (21.21)	27 (24.77)
<b>30-day mortality</b>	12 (27.91)	13 (19.70)	25 (22.94)
<b>Length of hospital stay after culture (median days, IQR)</b>	12 (6 - 29)	12.5 (8 - 23)	12 (8 - 27)

201 <sup>a</sup>Invasive devices 48 hours before or at the time of culture sampling

202 <sup>b</sup>In the previous year

203 <sup>c</sup>In the previous six months

204 **Phenotypic and molecular detection of carbapenemases.** 43 strains were found to harbor  
205 *bla*<sub>KPC</sub>, including *bla*<sub>KPC-2</sub> (83.72%) and *bla*<sub>KPC-3</sub> (6.97%), with most of them in the Tn4401  
206 isoform b (41/43); one isolate carried Tn4401 isoform a and the remaining isolate was  
207 negative for Tn4401. No other carbapenemase genes (*bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub>)  
208 were detected. Notably, a higher frequency of non-carbapenemase-producing *E. cloacae*  
209 (non-CP-Ecl) (66/109, 60.55%) were detected among CR-Ecl. In addition, a high rate of  
210 false-positive results were observed in the three-dimensional test (n=104, 95.41%),  
211 although only 39.55% of isolates harbored *bla*<sub>KPC</sub>. Similarly, MHT was positive in 38/65  
212 (58.46%) isolates, but only 19 (50%) harbored *bla*<sub>KPC</sub>.

213

214 **Clinical and microbiological description of infections caused by non- carbapenemase-**  
215 **producing *E. cloacae*.**

216 Patients infected with non-CP-Ecl presented mainly with surgical site infections (n=18,  
217 27.27%), followed by urinary tract infections (n=13, 19.70%) and intra-abdominal  
218 infections (n=11, 19.70%). In hospital mortality was 21.21% (n=14) and length of hospital  
219 stay after positive culture was 12.5 days (IQR 8-23). Patients were treated frequently with  
220 monotherapy (n=32, 48.48%), mostly with carbapenems (18/32) or fluoroquinolones  
221 (5/32). Combined therapy was administered in 27.27% (n=18) of patients, mainly  
222 carbapenems plus aminoglycosides (4/18), glycolcyclines (3/18) and polymixins (3/18).  
223 Additional characteristics of patients are presented in Table 1.

224

225 Non-CP-Ecl frequently harbored chromosomal  $\beta$ -lactamases gene *bla*<sub>ACT/MIR</sub> (n=32,  
226 53.33%) and *bla*<sub>ACT/MIR</sub>+*bla*<sub>TEM-1</sub> (n=11, 18.33%). Interestingly, additional analysis of  
227 *ompF* revealed 37/66 isolates had premature stop codons: D166X (20/37), E65X (10/37),

228 F145X (4/37), K3X (1/37), Q93X (1/37) and L105X (1/37). Meanwhile, 17/66 isolates had  
229 frameshift mutations and 23/66 had missense mutations (V141A and V141K) in loop 3,  
230 which constitutes the eyelet of the porin channel. In total 55/66 isolates had at least one of  
231 the above described mutations in the *ompF* sequence.

232

233 Of note, non-CP-Ecl exhibited high frequency of ertapenem resistance (85.94%), but lower  
234 resistance to other carbapenems (imipenem 19.35% and meropenem 3.03%). Resistance to  
235 gentamicin, ciprofloxacin and tigecycline were also frequently observed in non-CP-Ecl, but  
236 most isolates were susceptible to colistin (96.23%) (Table 2). The antibiotic resistance  
237 profile showed that most non-CP-Ecl were resistant to  
238 ertapenem+amikacina+gentamicin+ciprofloxacin (n=21, 31.82%).



239 Table 2. Microbiological characteristics of carbapenem-resistant *Enterobacter cloacae* complex isolates.

Isolate characterization	CP-Ecl n=43	Non-CP-Ecl n=66	Total isolates n=109
Threedimensional test	43 (100)	61 (92.42)	104 (95.41)
Antimicrobial resistance pattern			
Ceftazidime	37 (92.50)	47 (78.33)	84 (84.00)
Ceftriaxone	33 (94.29)	42 (84.00)	75 (88.24)
Cefepime	27 (65.85)	13 (20.00)	40 (37.74)
Ertapenem	33 (91.67)	55 (85.94)	88 (88.00)
Imipenem	40 (93.02)	12 (19.35)	52 (49.52)
Meropenem	38 (88.37)	2 (3.03)	40 (36.70)
Gentamicin	26 (60.47)	39 (60.00)	65 (60.19)
Amikacin	21 (48.84)	8 (12.12)	29 (26.61)
Ciprofloxacin	27 (62.79)	44 (66.67)	71 (65.14)
Tigecycline	29 (78.38)	36 (75.00)	65 (76.47)

Colistin	2 (6.25)	2 (3.77)	4 (4.71)
Multidrug resistant	21 (70.00)	33 (73.33)	54 (72.00)

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240

241 Bayesian analysis of *hsp60* sequences of 54 out of 66 isolates non-CP-Ecl strains revealed  
242 most isolates belonged to cluster VII/VIII (45/54), followed by cluster VI (4/54) (Figure 1  
243 and 3). Previous phylogenomics analysis, including several isolates from this study  
244 (EH005, EH012, EL012 and EP004), assigned cluster VII/VIII strains as *E. hormaechi*  
245 subsp. *steigerwaltii*. Only eight (8/53, 15.1%) isolates from this cluster carried KPC-2. Of  
246 note, 44/45 non-CP-Ecl isolates from this cluster had the following mutations in *ompF*:  
247 premature stop codons, mainly D166X (20/45), E65X (10/45) and F145X (4/45),  
248 frameshifts in the protein sequence (9/45) and alterations in the loop3 of the porin channel  
249 (V141A, 10/45), suggesting the carbapenem resistance in this cluster may be associated  
250 with the OmpF dysfunction. Additionally, PFGE analysis of 34 isolates from cluster  
251 VII/VIII revealed 12 isolates were closely related (Dice's coefficient >80%) (Figure 2,  
252 Table S1).

253

254

255 Figure 1. Bayesian phylogenetic tree depicting the relationship among *E. cloacae* complex  
256 isolates based on *hsp60* sequences obtained from 91 study isolates and 27 *hsp60* sequences  
257 of reference strains retrieved from GenBank. The Bayesian tree was constructed using the  
258 TMP3+I+G nucleotide substitution model. The strain *E. aerogenes* NBRC13534 was used  
259 as outgroup. Posterior probabilities are shown in each node. A high posterior probability  
260 support (>80%) was found for the clades with isolates from cluster I, cluster II, cluster IV,  
261 cluster VI/VII/VIII and cluster XI. A low posterior probability support was found for clades  
262 with *E. hormaechi* subspecies (54 and 53%). Isolates belonging to the three largest clusters  
263 are shown in bold.

264

265

266 Figure 2. PFGE dendrogram showing the genetic relationship among 68 isolates of  
267 carbapenem-resistant *E. cloacae* complex. The Dice similarity coefficient and the  
268 unweighted pair group method with arithmetic averages were used for dendrogram  
269 generation in Bionumerics software. Two groups of isolates were closely related according  
270 to the Dice coefficient (>80%); the first group were mainly KPC-negative and correspond  
271 mostly to cluster VII/VIII by *hsp60* phylogenetic analysis, the second group are mainly  
272 KPC-positive, and correspond primarily to cluster XI by *hsp60* analysis.

273

274

275 Among non-CP-Ecl, Hospital C accounted for 32/45 of cluster VII/VIII isolates, followed  
276 by Hospital A (9/45). PFGE pulsotypes were closely related among non-CP-Ecl isolates  
277 from Hospital C, suggesting a clonal dissemination of the similar non-CP-Ecl in this  
278 hospital (Figure 2 and 3). The majority of isolates from cluster VII/VIII were recovered  
279 spanning the two-year study period (Figure S2). Isolates from this cluster were found in a  
280 variety of infections (Figure S1).

281

282

283 Figure 3. (A) Distribution of *hsp60* clusters among KPC and non-KPC-Ecl isolates. (B)  
284 PFGE pulsotypes identified within cluster XI (CP-Ecl) and (C) cluster VII/VIII (non-CP-  
285 Ecl) isolates.

286 **Clinical and microbiological description of infections caused by carbapenemase-producing *E.***

287 *cloacae*.

288

289 Patients infected with CP-Ecl presented diverse infections including surgical site (n=7, 16.28%), intra-  
290 abdominal (n=7, 16.28%), primary bloodstream (n=7, 16.28%) and urinary tract infections (n=5,  
291 11.63%). The targeted treatment of CP-Ecl infected patients was predominantly monotherapy (n=16,  
292 37.21%), with tigecycline (4/16) or polymixins (5/16).

293 Regarding microbiological characteristics, CP-Ecl frequently carried additional  $\beta$ -lactamases, such as

294 *bla*<sub>ACT/MIR</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM-1</sub> and *bla*<sub>SHV-12</sub>. The most frequent  $\beta$ -lactamase profile was

295 *bla*<sub>ACT/MIR</sub>+*bla*<sub>KPC-2</sub> (n=10, 25.00%).

296 Susceptibility testing showed CP-Ecl isolates had high frequency of resistance to different antibiotics,

297 including cefepime (65.85%), imipenem (93.02%), meropenem (88.37%) and amikacin (48.84%), but

298 were susceptible to colistin with a MIC  $\leq$  0.5  $\mu$ g/mL (93.75%) (Table 2). The most common profile in

299 KPC-Ecl was resistance to all antibiotics except colistin.

300

301 Bayesian phylogenetic analysis of *hsp60* from 34 CP-Ecl isolates revealed most strains belonged

302 primarily to cluster VI (12/34) and XI (12/34) (Figure 1 and 3). Cluster VI includes *E. hormaechi*

303 subsp. *oharae* and the recently described *E. xiangfangensis*. Previous phylogenomics analysis of two

304 isolates (EH015 and EL036) from this cluster identified them as *E. xiangfangensis* (26). Most isolates

305 of cluster VI harbored KPC-2 (11/12) and were genetically diverse according PFGE (Figure 1 and 3).

306

307 Remarkably, 10/12 cluster XI isolates were closely related according PFGE analysis (Dice coefficient

308  $>$ 80%) (Figure 2, Table S2), 11/12 harbored KPC-2 and all harbored the same insertion in *ompF* (VT in

309 pos. 45) and the premature stop codon (L105X). Most KPC-positive infected patients were from

310 Hospital C (17/34), from which seven belonged to cluster XI. Cluster XI isolates caused mainly UTI  
311 and intra-abdominal infections (Figure S1).

312  
313 **Comparison of clinical characteristics between patients infected by carbapenemase-producing**  
314 **and non- carbapenemase producing *Enterobacter cloacae* complex.**

315 Patients infected with CP-Ecl and non-CP-Ecl were identified along the two years of the study with no  
316 cluster of cases over time (Figure S2). CP-Ecl patients were frequently hospitalized in the ICU (37.21%  
317 vs 12.12%) and had at least one medical device at the time or 48 hours before sampling, such as  
318 mechanical ventilation (30.23% vs 7.58%) and central venous catheter (41.86% vs. 21.21%) In  
319 addition, CP-Ecl infected patients had frequent exposure to carbapenems (39.53%) compared to non-  
320 CP-Ecl infected patients (15.15%).

321  
322 All-cause in-hospital mortality was higher among CP-Ecl (30.23%) than in non-CP-Ecl (21.21%)  
323 among infected patients. Similar findings were observed for all-cause 30-day mortality (27.91% in CP-  
324 Ecl and 19.70% in non-CP-Ecl). The median time to death was 6 days (IQR 3-11) in CP-Ecl and 9 days  
325 (IQR 3-14) in non-CP-Ecl infected patients.

326

327 **Discussion.**

328 Colombia is regarded as one of the KPC endemic regions, and approximately 80 to 86% of  
329 carbapenem-resistant isolates carried KPC carbapenemases, mostly in *K. pneumoniae* (12,27). Limited  
330 reports about CR-Ecl were available in the country. The national surveillance system reported in 2015  
331 that most (66.1%) carbapenem-non-susceptible *E. cloacae* isolates carried KPC (144/218), followed by  
332 KPC+GES (7/218), VIM (5/218), NDM (4/218), KPC+VIM (2/218) and GES (1/218). By contrast,  
333 25.22% (55/218) of carbapenem-non-susceptible *E. cloacae* did not harbor any carbapenemase (12). A

334 similar study conducted in eight Colombian regions showed that most CR-Ecl isolates (19/28, 67.85%)  
335 harbored KPC (28). By contrast, our study uncovered a significantly higher proportion (60.55%) of  
336 non-CP-Ecl in CR-Ecl.

337

338 In our study a higher proportion of isolates (60.55%), did not harbor carbapenemases (KPC, NDM,  
339 VIM, IMP or OXA-48), and remarkably, *hsp60* phylogenetic analysis revealed the predominance of  
340 cluster VII/VIII in non-CP-Ecl isolates. In addition, PFGE showed a predominant pulsotype within the  
341 non-CP-Ecl cluster VII/VIII, suggesting a clonal spread of the same strain in at least 12 patients.

342 Previous phylogenomics analysis using core SNPs and average nucleotide identity (ANI) confirmed  
343 identification of four isolates from this cluster as *E. hormaechi* subsp. *steigerwaltii*. In contrast to  
344 previous reports reporting genetic diversity among non-CP-Ecl (29), our findings demonstrated the  
345 clonal dissemination of non-CP-Ecl strains.

346

347 Carbapenem resistance in non-CP-Ecl isolates could be the result of  $\beta$ -lactamase production and porin  
348 loss. Pecora *et al.* (30), found that 32.4 to 52.3% of *E. cloacae* isolates not carrying carbapenemases  
349 genes but  $\beta$ -lactamase genes (CTX-M-15, TEM-116, AmpC) in conjunction with OmpC and OmpF  
350 defects, were resistant to carbapenems. This agreed with our results where a high rate of false-positives  
351 in the MHT and three-dimensional test was detected in non-CP-Ecl isolates. In fact, Wang *et al.* (31)  
352 reported 3.3% of false positive in non-carbapenemase producing but ESBL-producing  
353 Enterobacteriaceae, mainly CTX-M producers, indicating the lack of specificity of MHT for detection  
354 of serine carbapenemases. In our study, CTX-M-15 was only detected in 8 out of 66 non-CP-Ecl. In  
355 addition, these isolates also carried SHV-12 and SHV-27 ESBLs (10/66) and several mutations and  
356 premature stop codons in *ompF*. It is also important to highlight that in our study only 2/66 of non-CP-

357 Ecl and 38/43 CP-Ecl were resistant to meropenem, suggesting meropenem resistance may be useful  
358 for suspecting the presence of KPC in *E. cloacae* complex.

359

360 In our study, ~40% of the isolates were carbapenemase-producers, with 83.72% harboring KPC-2 and  
361 6.97% harboring KPC-3. Similarly, carbapenem resistance in *E. cloacae* is also associated with the  
362 presence of KPC in the USA, where outbreaks of closely related isolates of *E. cloacae* clone ST171  
363 harboring KPC-3 have been described in Minnesota and in North Dakota (8,32). In our study, a small  
364 outbreak of CP-Ecl harboring KPC-2 occurring in one of the hospitals was also detected. *hsp60*  
365 phylogenetic analysis revealed among CP-Ecl the clusters VI, XI and VII/VIII of the Hoffman and  
366 Roggenkamp scheme (22), but the majority of isolates from cluster XI, which comprises *E. cloacae*  
367 subsp. *cloacae*, shared the same pulsotype, suggesting transmission of the CP-Ecl strain in at least ten  
368 patients. Cluster VI and VII/VIII was also predominant in CP-Ecl isolates, however pulsotypes were  
369 diverse within these groups. These results suggest that in addition to clonal spread, horizontal transfer  
370 of KPC plasmids in diverse genetic backgrounds are important mechanisms in dissemination of CP-Ecl  
371 in our setting.

372

373 Noteworthy, CP-Ecl and non-CP-Ecl displayed distinct clinical characteristics and resistance patterns.  
374 Overall, non-CP-Ecl infected patients were hospitalized in general wards, while CP-Ecl patients were  
375 frequently hospitalized in the ICU, had mechanical ventilation, central venous catheter, vascular  
376 dialysis catheter and previous carbapenem exposure. Importantly, observed all-cause mortality (30.23%  
377 vs 21.21%, respectively) and 30-day mortality (27.91% vs 19.70%, respectively) was higher in CP-Ecl  
378 group compared to non-CP-Ecl. Other studies have reported a comparable mortality rate in patients  
379 infected with KPC-Ecl, close to 35% (7/20), with 15% (3/20) of deaths attributable to CRE infections  
380 (7). It is important to highlight that ICU stay at the time of sampling and previous use of carbapenems



381 were more frequent in the CP-Ecl infected group than in the non-CP-Ecl group. In support of these  
382 findings a previous study reported that ICU admission at the time of infection was common in CP-Ecl  
383 infections (7) and recently, Okamoto *et al.* (33) found that exposure to carbapenems (OR, 2.25; 95%  
384 CI, 1.06–4.77) was an independent risk factor for KPC-producing Gram-negative acquisition, in  
385 addition to colonization pressure (OR, 1.02; 95% CI, 1.01–1.04) and comorbidities measured by the  
386 Charlson index (OR, 1.14; 95% CI, 1.01–1.29). Although limited studies on CP-Ecl epidemiology are  
387 available, study addressing the identification of factors associated with ESBL enzymes in *E. cloacae*  
388 infections showed that the previous use of antibiotics (46/70 vs 17/20) in addition to mechanical  
389 ventilation (47/70 vs 19/20), were frequent in ESBL-positive-Ecl compared to ESBL-negative-Ecl  
390 infected patients, respectively (34). These results agreed with the preponderant role of carbapenems  
391 (33,35–37) and ICU stay (38–40) as risk factors for KPC-*K. pneumoniae* infection.

392  
393 This study has limitations. First, description of clinical and microbiological variables such as the  
394 colonization status of CR-Ecl patients was not included because it was missing for 42.20% of patients  
395 included in the study. Similarly, results from the MHT were only described for 65 isolates with  
396 available information. In addition, ompC sequencing analysis were not included in this study, due to  
397 the high diversity of ompC sequences among different clusters.

398  
399 In conclusion, our study revealed important differences in the molecular epidemiology of CR-Ecl in a  
400 KPC endemic setting, where non-CP-Ecl accounted for the majority of CR-Ecl infections. Both clonal  
401 spread and plasmid transfer are involved in CP-Ecl infections, while clonal spread was found the major  
402 mechanism in dissemination of non-CP-Ecl. Overall both CP-Ecl and non-CP-Ecl infections have  
403 similar clinical characteristics, but CP-Ecl infected patients had high mortality, were frequently  
404 hospitalized in ICU, have invasive devices and previous carbapenem exposure. The MHT and three-

405 dimensional test showed false positives for carbapenemase detection, but resistance to meropenem  
406 instead of ertapenem could help in the detection of CP-Ecl. Finally, the study highlights significant  
407 contribution of non-CP-Ecl to the overall prevalence of CR-Ecl. Infection control measures to curtail  
408 dissemination of CR-Ecl should not only focus on CP-Ecl but also include non-CP-Ecl.

409

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414

415 **Conflict of Interest.** The authors declare that they have no conflict of interest.

416

417

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555

### 556 **Supporting Information.**

557 **Table S1.** Clinical characteristics of patients infected with non-CP-Ecl isolates closely related by PFGE  
558 (Dice's coefficient >80) from cluster VII/VIII.

559 **Table S2.** Clinical characteristics of patients infected with CP-Ecl isolates closely related by PFGE  
560 (Dice's coefficient >80) from cluster XI.

561 **Figure S1.** Type of infections caused by CP-Ecl and non-CP-Ecl according to hsp60 clusters. (A) CP-  
562 Ecl isolates and (B) Non-CP-Ecl isolates.

563 **Figure S2.** Epidemic curve of carbapenem-resistant *E. cloacae* complex infected patients (A) according  
564 *E. cloacae* genetic cluster of Hoffman & Roggenkamp in Hospital C and (B) according KPC and non-  
565 KPC harboring *E. cloacae*.



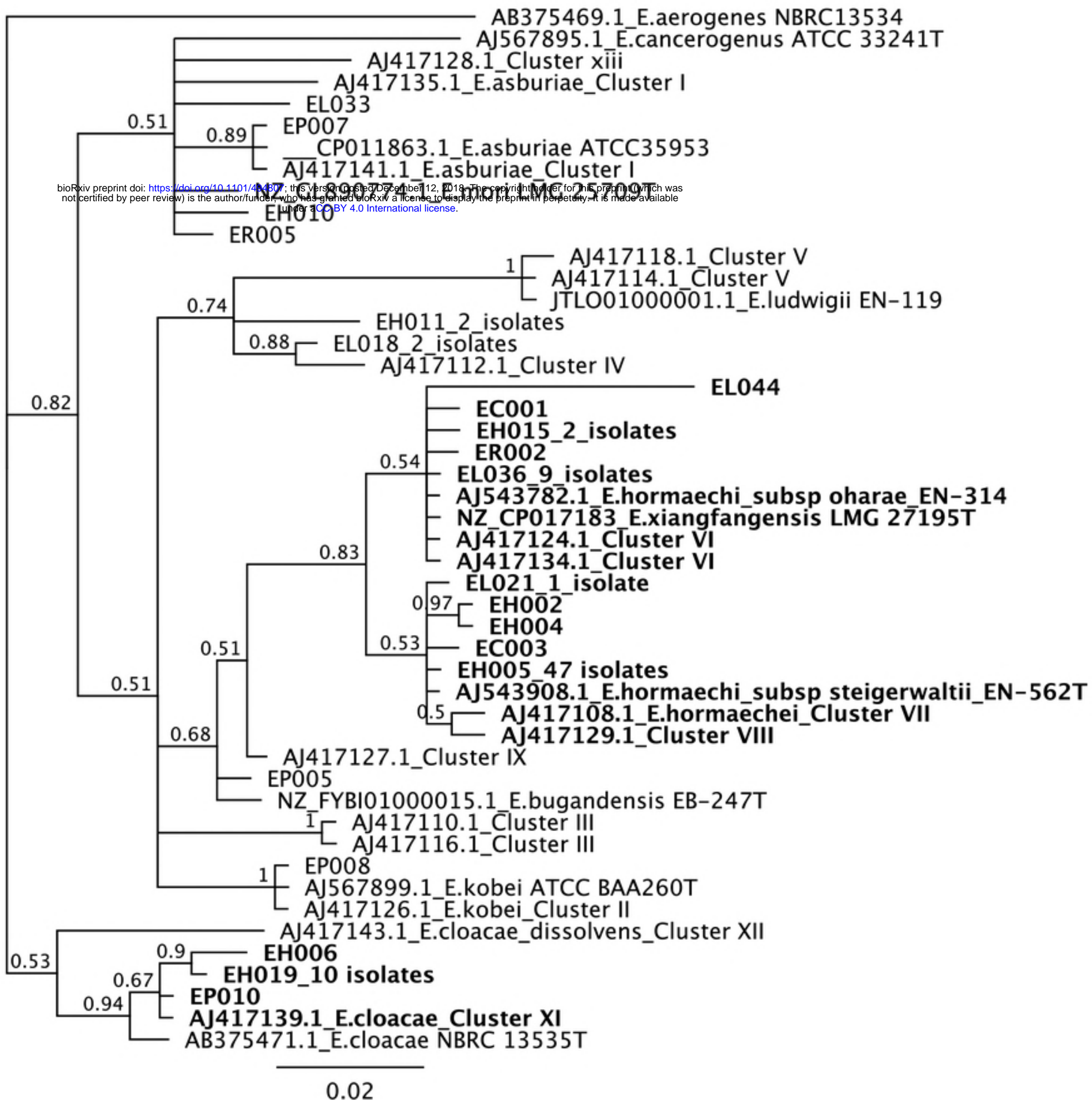


Figure 1

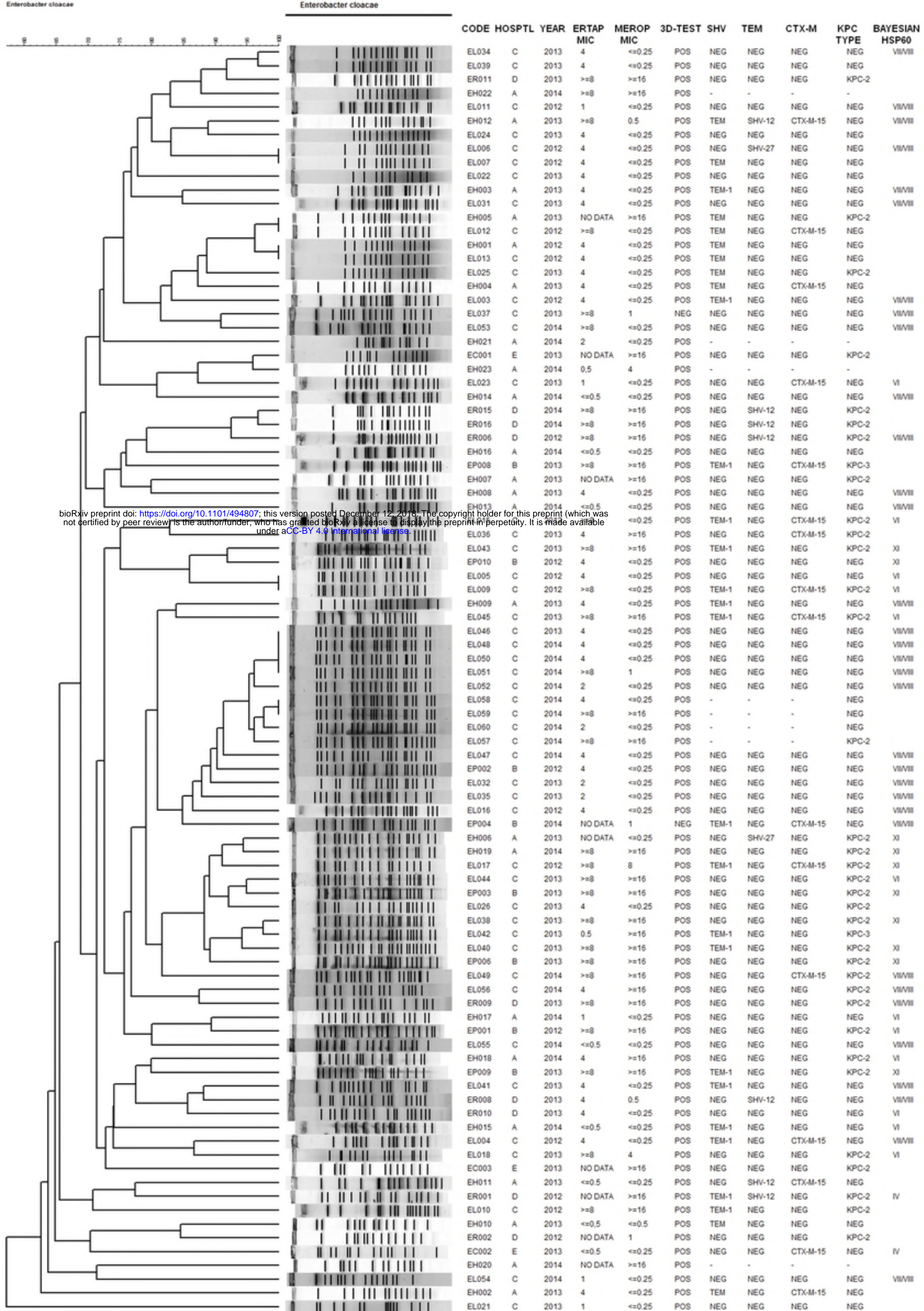


Figure 2