Whole-Genome Sequencing-Based Species Classification, Multilocus Sequence Typing, and Antimicrobial Resistance Mechanism Analysis of the Enterobacter cloacae Complex in Southern China

Xu Dong,a,b Mei Zhu,c Yaxuan Li,d Dawei Huang,e Lan Wang,d Chunxia Yan,d Linhua Zhang,e Fubo Dong,e Junwan Lu,d Xi Lin,b Keweili,b Qiyu Bao,b,d Cheng Cong,a Wei Pan e

a School of Medicine, Lishui University, Lishui, Zhejiang, China
b School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China
c Department of Clinical Laboratory, Zhejiang Hospital, Hangzhou, Zhejiang, China
d Medical Molecular Biology Laboratory, School of Medicine, Jinhua Polytechnic, Jinhua, Zhejiang, China
e The People’s Hospital of Yuhuan, Yuhuan, Zhejiang, China

Xu Dong and Mei Zhu contributed equally to this work. The order was determined by the corresponding author after negotiation.

ABSTRACT Members of the Enterobacter cloacae complex (ECC) are important opportunistic nosocomial pathogens that are associated with a great variety of infections. Due to limited data on the genome-based classification of species and investigation of resistance mechanisms, in this work, we collected 172 clinical ECC isolates between 2019 and 2020 from three hospitals in Zhejiang, China and performed a retrospective whole-genome sequencing to analyze their population structure and drug resistance mechanisms. Of the 172 ECC isolates, 160 belonged to 9 classified species, and 12 belonged to unclassified species based on ANI analysis. Most isolates belonged to E. hormaechei (45.14%) followed by E. kobei (13.71%), which contained 126 STs, including 62 novel STs, as determined by multilocus sequence typing (MLST) analysis. Pan-genome analysis of the two ECC species showed that they have an “open” tendency, which indicated that their Pan-genome increased considerably with the addition of new genomes. A total of 80 resistance genes associated with 11 antimicrobial agent categories were identified in the genomes of all the isolates. The most prevailing resistance genes (12/29, 41.38%) were related to β-lactams followed by aminoglycosides. A total of 247 β-lactamase genes were identified, of which the blaACT genes were the most dominant (145/247, 58.70%), followed by the blaTEM genes (21/247, 8.50%). The inherent ACT type β-lactamase genes differed among different species. blaACT-2 and blaACT-3 were only present in E. asburiae, while blaACT-9, blaACT-12, and blaACT-6 exclusively appeared in E. kobei, E. ludwigi, and E. mori. Among the six carbapenemase-encoding genes (blaNDM-1, blaNDM-5, blaIMP-1, blaIMP-4, blaIMP-26, and blaKPC-3) identified, two (blaNDM-1 and blaIMP-1) were identified in an ST78 E. hormaechei isolate. Comparative genomic analysis of the carbapenemase gene-related sequences was performed, and the corresponding genetic structure of these resistance genes was analyzed. Genome-wide molecular characterization of the ECC population and resistance mechanism would offer valuable insights into the effective management of ECC infection in clinical settings.

IMPORTANCE The presence and emergence of multiple species/subspecies of ECC have led to diversity and complications at the taxonomic level, which impedes our further understanding of the epidemiology and clinical significance of species/subspecies of ECC. Accurate identification of ECC species is extremely important. Also, it is of great importance to study the carbapenem-resistant genes in ECC and to further understand the mechanism of horizontal transfer of the resistance genes by
an analyzing the surrounding environment around the genes. The occurrence of ECC carrying two MBL genes also indicates that the selection pressure of bacteria is further increased, suggesting that we need to pay special attention to the emergence of such bacteria in the clinic.

**KEYWORDS** Enterobacter cloacae complex, whole-genome sequencing-based species classification, MLST, antimicrobial resistance mechanism, Pan-genome

The Enterobacter cloacae complex (ECC) is one of the most common nosocomial pathogens causing healthcare-associated infections involving pneumonia, urinary tract infections, and septicemia (1). Previous studies have reported that the *E. cloacae* complex mainly comprises six species, including *E. cloacae*, Enterobacter asburiae, Enterobacter hormaechei, Enterobacter kobei, Enterobacter ludwigi, and Enterobacter nimipressuralis (2). Among them, *E. hormaechei* and *E. cloacae* are most frequently isolated from human clinical specimens (3). Based on the rapid development of next-generation sequencing, the most widely used method of species identification, *hsp60* typing, could be replaced by comparison of the sequenced *Enterobacter* genomes through whole-genome sequencing (WGS), which provides higher resolution to distinguish the *E. cloacae* complex at the taxonomic level. The ECC has been further classified into clades (A to V), including the Hoffmann cluster (I to XII) (4, 5).

Most isolates of the ECC produce the chromosomally encoded AmpC β-lactamase and intrinsically exhibit resistance to several antimicrobial agents, such as ampicillin, amoxicillin, amoxicillin-clavulanic acid, and first- and second-generation cephalosporins, whereas they are generally susceptible to fluoroquinolones, chloramphenicol, aminoglycosides, tetracyclines, trimethoprim-sulfamethoxazole, piperacillin-tazobactam, and carbapenems (2). Due to the selective pressure of antimicrobials in the clinic, an increasing number of ECC isolates carrying different acquired resistance genes have been detected. The ECC has become the third major drug-resistant *Enterobacteriaceae* species associated with nosocomial infections following *Escherichia coli* and *Klebsiella pneumoniae* due to the prevalence of Extended spectrum β-lactamases (ESBLs) and carbapenemases in the constituent species (6). The most clinically prevalent ESBLs are TEM, SHV, and CTX-M enzymes (7), and the major types of carbapenemases are KPC, NDM, and IMP/VIM (8). In China, the first reported carbapenemase in *Enterobacteriaceae* was KPC-2, identified in Shanghai in 2007 (9), and more types of carbapenemases (IMP, VIM, and NDM) have been identified in different geographical regions (10–12). The acquisition of these genes is most often associated with mobile genetic elements (MGEs), such as plasmids and transposons, that can be easily transferred between different species.

In this work, we performed a retrospective whole-genome sequencing to analyze the population structure and the distribution of resistance genes, especially focusing on resistance genes more concerned in the clinic (ESBLs and carbapenemases), among 172 clinical *E. cloacae* complex isolates collected between 2019 and 2020 from Zhejiang, China. We observed and identified a novel isolate that harbored two metallobeta-lactamases (MBLs).

**RESULTS**

**Characteristics of clinical ECC isolates.** A total of 172 ECC strains were isolated from different sources: wound secretion (*n* = 55), sputum (*n* = 46), urine (*n* = 21), throat swab (*n* = 13), blood (*n* = 11), pus (*n* = 10), bile (*n* = 5), ascites (*n* = 3), bronchoalveolar lavage (*n* = 2), tissue (*n* = 2), drainage (*n* = 2), catheter (*n* = 1), and semen (*n* = 1). The average nucleotide identity (ANI) results of 172 isolates revealed that the predominant species was *E. hormaechei* (*n* = 79) followed by *E. kobei* (*n* = 24), *E. rogenkampfi* (*n* = 15), *E. asburiae* (*n* = 14), *E. bugandensis* (*n* = 13), *E. cloacae* (*n* = 10), *E. ludwigi* (*n* = 3), and *E. mori* (*n* = 3). Twelve isolates with ANI values below the threshold (95%) for the classified species/subspecies were grouped into 4 clades (L, O, P, and T) (Table 1; Fig. 1). These 172 ECC isolates exhibited high susceptibility to amikacin (98.8%), gentamicin (93.6%), meropenem (90.1%), ciprofloxacin (78.5%), cefepime (77.3%), tigecycline (76.7%), ceftazidime (71.5%), chloramphenicol (68.6%), fosfomycin (59.9%), and tetracycline (54.7%) and low susceptibility to...
| Strain | Species | Clade | hsp60 typing | ST  | Location  |
|--------|---------|-------|--------------|-----|-----------|
| 1      | E. asburiae | J     | I            | 1682-NEW | Hangzhou  |
| 2      | E. hormaechei subsp. steigerwaltii | B     | VIII         | 110  | Hangzhou  |
| 4      | E. hormaechei subsp. xiangfangensis | A     | VI           | 511  | Hangzhou  |
| 5      | E. hormaechei subsp. steigerwaltii | B     | VIII         | 112  | Hangzhou  |
| 6      | E. hormaechei subsp. steigerwaltii | B     | VIII         | 461  | Hangzhou  |
| 7      | E. hormaechei subsp. xiangfangensis | A     | VI           | 1707-NEW | Hangzhou |
| 9      | E. ludwigii | I     | V            | 1708-NEW | Hangzhou |
| 10     | E. kobei   | Q     | II           | 56   | Hangzhou  |
| 11     | E. kobei   | Q     | II           | 56   | Hangzhou  |
| 12     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 133  | Hangzhou  |
| 13     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1683-NEW | Hangzhou |
| 14     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 110  | Hangzhou  |
| 15     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 110  | Hangzhou  |
| 16     | E. kobei   | Q     | II           | 125  | Hangzhou  |
| 19     | E. rogenkampii | M   | IV           | 1681-NEW | Hangzhou |
| 20     | E. rogenkampii | M   | IV           | 501  | Hangzhou  |
| 21     | E. kobei   | Q     | II           | 365  | Hangzhou  |
| 22     | E. rogenkampii | M   | IV           | 1685-NEW | Hangzhou |
| 23     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 550  | Hangzhou  |
| 24     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 536  | Hangzhou  |
| 25     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 175  | Hangzhou  |
| 27     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 45   | Hangzhou  |
| 28     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 776  | Hangzhou  |
| 31     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 46   | Hangzhou  |
| 32     | E. asburiae | J     | I            | 1692-NEW | Hangzhou |
| 33     | E. hormaechei subsp. hoffmannii | D     | III          | 78   | Hangzhou  |
| 34     | E. kobei   | Q     | II           | 56   | Hangzhou  |
| 35     | E. kobei   | Q     | II           | 423  | Hangzhou  |
| 36     | E. ludwigii | I     | V            | 1693-NEW | Hangzhou |
| 37     | E. bugandensis | R | IX          | 1694-NEW | Hangzhou |
| 38     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1695-NEW | Hangzhou |
| 39     | E. hormaechei subsp. hoffmannii | D     | III          | 78   | Hangzhou  |
| 41     | E. hormaechei subsp. hoffmannii | D     | III          | 78   | Hangzhou  |
| 42     | E. hormaechei subsp. xiangfangensis | A     | VI           | 1696-NEW | Hangzhou |
| 43     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 50   | Hangzhou  |
| 44     | E. rogenkampii | M   | IV           | 1697-NEW | Hangzhou |
| 45     | E. kobei   | Q     | II           | 125  | Hangzhou  |
| 46     | E. asburiae | J     | I            | 252  | Hangzhou  |
| 49     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1698-NEW | Hangzhou |
| 50     | E. mori    | F     |             | 1699-NEW | Hangzhou |
| 51     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1698-NEW | Hangzhou |
| 52     | E. hormaechei subsp. hoffmannii | D     | III          | 78   | Hangzhou  |
| 53     | E. hormaechei subsp. xiangfangensis | A     | VI           | 114  | Hangzhou  |
| 54     | E. rogenkampii | M   | IV           | 1700-NEW | Hangzhou |
| 55     | E. rogenkampii | E     | VII          | 269  | Hangzhou  |
| 56     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1698-NEW | Hangzhou |
| 57     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1711-NEW | Hangzhou |
| 58     | E. bugandensis | R | IX          | 1701-NEW | Hangzhou |
| 59     | E. hormaechei subsp. xiangfangensis | A     | VI           | 724  | Hangzhou  |
| 60     | E. cloacae complex | T  |             | 1702-NEW | Hangzhou |
| 61     | E. bugandensis | R | IX          | 718  | Hangzhou  |
| 62     | E. cloacae complex | T  |             | 1703-NEW | Hangzhou |
| 63     | E. bugandensis | T     |             | 1702-NEW | Hangzhou |
| 64     | E. cloacae complex | D  | III         | 78   | Hangzhou  |
| 65     | E. cloacae complex | T  |             | 1704-NEW | Hangzhou |
| 66     | E. bugandensis | R     | IX          | 718  | Hangzhou  |
| 67     | E. cloacae complex | L  |             | 414  | Hangzhou  |
| 68     | E. kobei   | Q     | II           | 1601-NEW | Hangzhou |
| 69     | E. mori    | F     |             | 1706-NEW | Hangzhou |
| 70     | E. hormaechei subsp. hoffmannii | D     | III          | 78   | Hangzhou  |
| 71     | E. hormaechei subsp. hoffmannii | D     | III          | 78   | Hangzhou  |

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TABLE 1 (Continued)

| Strain | Species | Clade | hsp60 typing | ST | Location   |
|--------|---------|-------|--------------|----|------------|
| 79     | E. roggenkampii | M     | IV           | 501 | Hangzhou  |
| 81     | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1698-NEW | Hangzhou  |
| 102    | E. hormaechei subsp. steigerwaltii | B     | VIII         | 554 | Wenzhou    |
| 103    | E. asburiae | J     | I            | 25  | Wenzhou    |
| 104    | E. cloacae complex | O     |              | 1647-NEW | Wenzhou    |
| 105    | E. asburiae | J     | I            | 1648-NEW | Wenzhou    |
| 106    | E. bugandensis | R     | IX           | 1651-NEW | Wenzhou    |
| 108    | E. cloacae complex | L     |              | 414 | Wenzhou    |
| 109    | E. kobei | Q     | II           | 280 | Wenzhou    |
| 110    | E. cloacae complex | L     |              | 414 | Wenzhou    |
| 111    | E. asburiae | J     | I            | 1649-NEW | Wenzhou    |
| 112    | E. bugandensis | R     | IX           | 1651-NEW | Wenzhou    |
| 114    | E. cloacae subsp. cloacae | G     | XI           | 1650-NEW | Wenzhou    |
| 115    | E. bugandensis | R     | IX           | 1651-NEW | Wenzhou    |
| 116    | E. roggenkampii | M     | IV           | 43  | Wenzhou    |
| 117    | E. roggenkampii | M     | IV           | 1652-NEW | Wenzhou    |
| 118    | E. cloacae subsp. dissolvens | H     | XII          | 1653-NEW | Wenzhou    |
| 120    | E. hormaechei subsp. xiangfangensis | A     | VI           | 120 | Wenzhou    |
| 121    | E. hormaechei subsp. xiangfangensis | A     | VI           | 136 | Wenzhou    |
| 122    | E. cloacae complex | L     |              | 414 | Wenzhou    |
| 123    | E. mori | F     |              | 1655-NEW | Wenzhou    |
| 125    | E. asburiae | J     | I            | 1656-NEW | Wenzhou    |
| 126    | E. kobei | Q     | II           | 563 | Wenzhou    |
| 127    | E. kobei | Q     | II           | 563 | Wenzhou    |
| 128    | E. hormaechei subsp. xiangfangensis | A     | VI           | 331 | Wenzhou    |
| 130    | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1657-NEW | Wenzhou    |
| 131    | E. hormaechei subsp. xiangfangensis | A     | VI           | 1658-NEW | Wenzhou    |
| 132    | E. asburiae | J     | I            | 317 | Wenzhou    |
| 133    | E. kobei | Q     | II           | 1661-NEW | Wenzhou    |
| 134    | E. cloacae subsp. dissolvens | H     | XII          | 1659-NEW | Wenzhou    |
| 135    | E. roggenkampii | M     | IV           | 1032 | Wenzhou     |
| 136    | E. cloacae subsp. cloacae | G     | XI           | 412 | Wenzhou    |
| 137    | E. hormaechei subsp. xiangfangensis | A     | VI           | 1660-NEW | Wenzhou    |
| 138    | E. hormaechei subsp. xiangfangensis | A     | VI           | 1660-NEW | Wenzhou    |
| 140    | E. hormaechei subsp. xiangfangensis | A     | VI           | 1660-NEW | Wenzhou    |
| 141    | E. cloacae subsp. cloacae | G     | XI           | 412 | Wenzhou    |
| 142    | E. cloacae subsp. cloacae | G     | XI           | 406 | Wenzhou    |
| 143    | E. kobei | Q     | II           | 1661-NEW | Wenzhou    |
| 144    | E. hormaechei subsp. xiangfangensis | A     | VI           | 1662-NEW | Wenzhou    |
| 145    | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1497-NEW | Wenzhou    |
| 146    | E. hormaechei subsp. xiangfangensis | A     | VI           | 127 | Wenzhou    |
| 147    | E. hormaechei subsp. xiangfangensis | A     | VI           | 182 | Wenzhou    |
| 148    | E. hormaechei subsp. xiangfangensis | A     | VI           | 1663-NEW | Wenzhou    |
| 149    | E. hormaechei subsp. steigerwaltii | B     | VIII         | 65  | Wenzhou    |
| 150    | E. kobei | Q     | II           | 32  | Wenzhou    |
| 151    | E. hormaechei subsp. steigerwaltii | B     | VIII         | 116 | Wenzhou    |
| 152    | E. hormaechei subsp. steigerwaltii | B     | VIII         | 1664-NEW | Wenzhou    |
| 153    | E. kobei | Q     | II           | 56  | Wenzhou    |
| 154    | E. cloacae subsp. cloacae | G     | XI           | 1665-NEW | Wenzhou    |
| 155    | E. hormaechei subsp. xiangfangensis | A     | VI           | 557 | Wenzhou    |
| 156    | E. bugandensis | R     | IX           | 1085 | Wenzhou   |
| 157    | E. roggenkampii | M     | IV           | 1666-NEW | Wenzhou    |
| 158    | E. cloacae complex | O     |              | 1667-NEW | Wenzhou    |
| 159    | E. hormaechei subsp. oharae | C     | VI           | 1668-NEW | Wenzhou    |
| 160    | E. asburiae | J     | I            | 53  | Wenzhou    |
| 161    | E. cloacae complex | L     |              | 1669-NEW | Wenzhou    |
| 162    | E. hormaechei subsp. xiangfangensis | A     | VI           | 407 | Wenzhou    |
| 163    | E. hormaechei subsp. xiangfangensis | A     | VI           | 264 | Wenzhou    |
| 164    | E. asburiae | J     | I            | 1670-NEW | Wenzhou    |
| 165    | E. cloacae complex | P     |              | 669 | Wenzhou    |
| 166    | E. hormaechei subsp. xiangfangensis | A     | VI           | 418 | Wenzhou    |
| 167    | E. asburiae | J     | I            | 610 | Wenzhou    |
| 168    | E. roggenkampii | M     | IV           | 1671-NEW | Wenzhou    |

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colistin (28.0%), ampicillin (0.6%), and cefoxitin (0%) (Table 2). Ten isolates (5.8%, 10/172) exhibited an ESBL-positive phenotype, most of which belonged to *E. hormaechei* \((n = 6)\), followed by *E. kobei* \((n = 2)\). Among the 14 isolates with carbapenem resistance phenotypes, *E. hormaechei* was the predominant species \((n = 9)\) followed by *E. kobei* \((n = 3)\).

**Distribution of resistance genes among ECC isolates.** A total of 80 antibiotic resistance genes (>80 similarity with the function characterized resistance genes) associated with 11 antimicrobial agent categories were identified among the genomes of all the isolates, such as aminoglycosides \((aph(6)-Id, aac(6’)-IIC, aac(6’)-Ib, aph(3’)-Ib, and aac (3)-Iia)\), beta-lactam \((bla{\text{A}}_{\text{KDO}}, \text{bla}{\text{A}}_{\text{PCG-2}}, \text{bla}{\text{D}}_{\text{OXH-M-15}}, \text{bla}{\text{C}}_{\text{CTX-M-15}}, \text{bla}{\text{MP}}, \text{bla}{\text{SHV-1}}, \text{bla}{\text{SHV-12}}, \text{fluoroquinolone} (\text{qnrE}1 \text{and qnrS2}), \text{fosfomycin} (\text{fosA}3), \text{macrolide (ereA}), \text{phenicol (catA2 and floR)}, \text{polymyxin} (\text{mcr-10}), \text{rifampicin} (\text{arr-6}), \text{sulfonamide (sul2)}, \text{tetracycline (tetA}), \text{and trimethoprim (dfrA12)}\) (Fig. S1 in Supplemental File 1). The most prevalent resistance

| Strain | Species | Clade | hsp60 typing | ST | Location |
|--------|---------|-------|--------------|----|----------|
| 169    | *E. roggenkampii* | M     | IV           | 501 | Wenzhou  |
| 170    | *E. cloacae subsp. cloacae* | G     | XI           | 1672-NEW | Wenzhou |
| 171    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 554 | Wenzhou  |
| 172    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 171 | Wenzhou  |
| 174    | *E. asburiae* | J     | I            | 1673-NEW | Wenzhou |
| 176    | *E. roggenkampii* | M     | IV           | 272 | Wenzhou  |
| 177    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 407 | Wenzhou  |
| 179    | *E. roggenkampii* | M     | IV           | 1674-NEW | Wenzhou |
| 180    | *E. bugandensis* | R     | IX           | 1675-NEW | Wenzhou |
| 181    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 171 | Wenzhou  |
| 182    | *E. kobei* | Q     | II           | 280 | Wenzhou  |
| 183    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 171 | Wenzhou  |
| 185    | *E. bugandensis* | R     | IX           | 1676-NEW | Wenzhou |
| 186    | *E. cloacae subsp. cloacae* | G     | XI           | 524 | Wenzhou  |
| 187    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 1116 | Wenzhou |
| 188    | *E. bugandensis* | R     | IX           | 1677-NEW | Wenzhou |
| 189    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 171 | Wenzhou  |
| 190    | *E. kobei* | Q     | II           | 125 | Wenzhou  |
| 191    | *E. cloacae subsp. cloacae* | G     | XI           | 1678-NEW | Wenzhou |
| 193    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 980  | Wenzhou  |
| 194    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 171  | Wenzhou  |
| 195    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 346  | Wenzhou  |
| 196    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 1679-NEW | Wenzhou |
| 197    | *E. kobei* | Q     | II           | 1680-NEW | Wenzhou |
| 198    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 171  | Wenzhou  |
| 199    | *E. kobei* | Q     | II           | 691  | Wenzhou  |
| 200    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 758  | Wenzhou  |
| 201    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 45   | Wenzhou  |
| 202    | *E. kobei* | Q     | II           | 1029 | Wenzhou  |
| 203    | *E. kobei* | Q     | II           | 1029 | Wenzhou  |
| 204    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 1683-NEW | Wenzhou |
| 205    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 127  | Wenzhou  |
| 206    | *E. roggenkampii* | M     | IV           | 501  | Wenzhou  |
| 207    | *E. asburiae* | J     | I            | 27   | Wenzhou  |
| 208    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 92   | Wenzhou  |
| 209    | *E. cloacae complex* | L     |              | 414  | Wenzhou  |
| 210    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 182  | Wenzhou  |
| 211    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 1684-NEW | Wenzhou |
| 212    | *E. kobei* | Q     | II           | 1243 | Wenzhou  |
| 302    | *E. kobei* | Q     | II           | 1687-NEW | Huzhou |
| 303    | *E. bugandensis* | R     | IX           | 599  | Huzhou  |
| 305    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 267  | Huzhou  |
| 306    | *E. hormaechei subsp. xiangfangensis* | A     | VI           | 1689-NEW | Huzhou |
| 307    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 1690-NEW | Huzhou |
| 308    | *E. kobei* | Q     | II           | 1691-NEW | Huzhou |
| 309    | *E. hormaechei subsp. steigerwaltii* | B     | VIII         | 93   | Huzhou  |
| 310    | *E. bugandensis* | R     | IX           | 718  | Huzhou  |
gene type belonged to β-lactams (30/80, 37.50%) followed by aminoglycosides (17/80, 21.25%).

A large number (247) of β-lactamase genes were identified in the 172 genomes. The \(\text{bla}_{\text{ACT}}\) genes were the most dominant (145/247, 58.70%) followed by the \(\text{bla}_{\text{TEM}}\) genes (21/247, 8.50%, \(\text{bla}_{\text{TEM-1D}}\) only), while the \(\text{bla}_{\text{KPC}}\) genes (1/247, 0.40%, \(\text{bla}_{\text{KPC-2}}\)) had the lowest frequency. \(\text{fosA}\) genes were found in most strains (153/172, 88.95%), covering all species except \(E.\) hormaechei subsp. hormaechei, \(E.\) hormaechei subsp. oharae, and \(E.\) ludwigii (Fig. S1 in Supplemental File 1). Given the widespread use of β-lactam antibiotics in clinical settings, in this work, we gave more attention to resistance genes encoding beta-lactamases, especially those acquired horizontally (Fig. 2). As shown in Fig. S1 in Supplemental File 1, the distribution of some inherent AmpCs differed among different species. For example, \(\text{bla}_{\text{ACT-2}}\) and \(\text{bla}_{\text{ACT-3}}\) were only present in \(E.\) asburiae, whereas \(\text{bla}_{\text{ACT-9}}, \text{bla}_{\text{ACT-12}},\) and \(\text{bla}_{\text{ACT-6}}\) were exclusively identified in \(E.\) kobei, \(E.\) ludwigii, and \(E.\) mori. Of note, ESBL genes and MBL genes were predominant among the horizontally acquired genes. ESBL genes were found in 26 isolates, with \(\text{bla}_{\text{TEM-1D}}\) being the most prevalent (\(n = 18\)) followed by \(\text{bla}_{\text{CTX-M-3}} (n = 8),\) and \(\text{bla}_{\text{SHV-12}} (n = 5).\) Carbapenem-resistant genotypes were present in 14 strains, with \(\text{bla}_{\text{NDM-5}}\) being the most prevalent (\(n = 6\)) followed by \(\text{bla}_{\text{NDM-1}} (n = 4), \text{bla}_{\text{IMP-4}} (n = 2), \text{bla}_{\text{IMP-1}} (n = 1), \text{bla}_{\text{IMP-26}} (n = 1),\) and \(\text{bla}_{\text{KPC-2}}\)
(n = 1). It was also interesting that among isolates carrying the ESBLs or carbapenem genes, only bla_NDM-5 showed some species specificity, appearing only in *E. hormaechei* subsp. *xiangfangensis*. Importantly, one isolate belonging to the *E. hormaechei* subsp. *hoffmannii* harbored two MBL genes, bla_NDM-1 and bla_IMP-1.

**MLST analysis.** MLST analysis showed that these 172 ECC isolates were divided into 126 STs, including 62 novel STs (ST1497, ST1601, ST1647-1653, ST1655-1685, ST1687, ST1689-1708, and ST1711) (Table 1). Among these STs, the predominant epidemic type was ST78 (n = 7), followed by ST171 (n = 6) and ST414 (n = 5) (Table 1; Fig. S2 in Supplemental File 1).

**Pan-genome analysis of *E. hormaechei* and *E. kobei.* Because of the high frequency of the acquired resistance genes in the two species *E. hormaechei* and *E. kobei*, a pan-genome analysis was performed to determine the dynamics of the bacterial genomes. The results showed that a total of 2,932 core genes, 7,879 accessory genes (genes present in two or more genomes), and 8,629 unique genes (a gene specific to a single genome (13)) were found in the *E. hormaechei* strains. Similarly, there were 3,603 core genes, 3,577 accessory genes, and 4,216 unique genes among the *E. kobei* strains (Fig. S3 in Supplemental File 1).

The rarefaction curve of the two species revealed that as genomes were sampled, the number of pan-genome genes did not show a trend of saturation, which indicated that the pan-genome of the two species were open according to the Heaps’ law model. The tendency for an open status, to some extent, meant that species with a higher number of pangenome genes had a greater capacity to acquire exogenous

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**TABLE 2** Susceptibility profiles and MICs for 172 ECC strains

| Antibiotics   | % Resistant | % Intermediate | % Susceptible | MIC<sub>50</sub> (mg/L) | MIC<sub>90</sub> (mg/L) | MIC range (mg/L) |
|---------------|-------------|----------------|---------------|--------------------------|--------------------------|------------------|
| Ampicillin    | 95.9        | 3.5            | 0.6           | 256                      | >1024                    | 1-1024           |
| Cefoxitin     | 99.4        | 0.6            | 0.0           | 512                      | 1024                     | 0.5-1024         |
| Cefazidime    | 25.0        | 3.5            | 71.5          | 0.25                     | 256                      | 0.125-256        |
| Cefepime      | 16.3        | 6.4            | 77.3          | 0.25                     | 32                       | 0.125-256        |
| Meropenem     | 8.1         | 1.7            | 90.1          | 0.125                    | 0.5                      | 0.0125-64        |
| Gentamicin    | 6.4         | 0.0            | 93.6          | 0.25                     | 2                        | 0.125-256        |
| Amikacin      | 1.2         | 0.0            | 98.8          | 1                        | 4                        | 0.5-512          |
| Ciprofloxacin | 14.5        | 7.0            | 78.5          | 0.125                    | 2                        | 0.125-256        |
| Chloramphenicol| 16.3   | 15.1          | 68.6          | 8                        | 256                      | 1-1024           |
| Fosfomycin    | 22.1        | 18.0           | 59.9          | 64                       | 256                      | 1-1024           |
| Colistin      | 71.5        | 28.5           | 0.0           | 16                       | 256                      | 0.125-256        |
| Tigecycline   | 3.5         | 19.8           | 76.7          | 1                        | 4                        | 0.125-256        |
| Tetracycline  | 18.0        | 27.3           | 54.7          | 4                        | 64                       | 1-256            |

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![FIG 2](image-url) Distributions of acquired β-lactamase genes in the 26 isolates. Blue and white squares represent the presence and absence of genes, respectively.
genes. However, *E. hormaechei* showed a lower number of core genes but a higher number of accessory and unique genes than *E. kobei*, which might be due to the diversity of *E. hormaechei* strains, which included more (5) subspecies.

To further understand the functional differences of pangenome genes, Cluster of Orthologous Groups of proteins (COG) functional classification of core genes, accessory genes, and unique genes of the two species was performed. The results indicated that the two species shared a highly similar COG category repartition of the core genomes, mainly associated with the functional classification of inorganic ion transport and metabolism, transcription, amino acid transport, and metabolism. Compared with the core genome, the unique and accessory genomes presented greater abundance in replication, recombination, and repair, which indicated that the functions of these genes are more likely to be associated with plasmid maintenance (14). Interestingly, there was a higher proportion of genes with unknown function (35.57% to 36.01%), indicating that the pan-genome genes of the two species have not been intensively studied yet.

**Genetic environment of carbapenem-resistant genes.** Six carbapenemase genes were identified in 14 ECC isolates, including blaNDM-1, blaNDM-5, blaIMP-1, blaIMP-4, blaIMP-26, and blaKPC-2. As shown in Fig. S4A in Supplemental File 1, the genetic context of blaKPC-2 in the IncR plasmid pECC207-88 was consistent with that of another *Enterobacter cloacae* IncR plasmid, pHN84KPC (KY296104). However, the transposase of the Tn3 unit upstream of blaKPC-2 differed from the classical Tn1722-blaKPC-2-unit transposon in the *Klebsiella pneumoniae* IncR plasmid pKP048 (FJ628167). Although the flanks of blaKPC-2 were enveloped by ISKpn6 and ISKpn27 in both pKP048 and pECC207-88, the transposon followed by ISKpn27 in pECC207-88 was an insertion sequence that shared 84% nucleotide similarity with ISEc63, a 4,473 bp element that belonged to the Tn3 family. Notably, similar ISEc63-like elements have also been reported in other *Klebsiella pneumoniae* strains with nonclassical transposon elements (15).

The genetic environments surrounding blaNDM-1 are shown in Fig. S4B in Supplemental File 1. Four plasmids carrying blaNDM-1 could be classified into different sequence types. The type A plasmid lacked the ISaba125 element upstream of IS5, and the genes downstream of groEL were different from those in the previously reported IncX3 plasmid pNDM-ECN49 (KP765744). The cutA1 gene of the type B plasmid was truncated by the insertion of IS26. Thus, the genes downstream of cutA1 changed greatly compared with the sequences of the type A plasmid. The transposase also exhibited a difference between IS3000 and ΔISaba125. Furthermore, similar to the type A plasmid, the sequence structure of type B lacked the ISaba125 element upstream of IS7 compared with the epidemic IncX3 plasmid pZH8-4 (CP059715). The genetic structure of the type C plasmid shared the highest similarity with the transposon Tn4600, which was composed of Tn6292 and a truncated Tn3000. However, compared with Tn6460, Tn6292 was inserted into the middle region of ΔTn3000 (dsbD gene) instead of the region downstream of ΔTn3000. Notably, no structure completely consistent with that of the type C plasmid was found in the NCBI nucleotide database. Six plasmids possessed the same genetic background of blaNDM-5 (IS3000-ISaba125-IS5-ΔISaba125-blaNDM-5×2-blaqacE-DsbC-DcutA1-IS26-umuD-ISKox3), which is the same as that of IncX3 plasmid pNDM-MGR194 (KF220657) from *Klebsiella pneumoniae* strain MGR194 isolated in India (Fig. S4C in Supplemental File 1).

All of the IMP genes were located in class 1 integrons (Fig. S4D in Supplemental File 1). The gene cassette of the blaIMP-26-containing integron was blaIMP-26-ltrA-qacEΔ1-sul1, which was the same as that of IncHI2 plasmid pMP26 from *E. cloaca* (MH399264). Two IncN plasmids (pECC46-54 and pECC79-52) carrying blaIMP-4 had a structurally similar genetic context except for the insertion of an ISSen4 between blaIMP-4 and ltrA. Moreover, the genes surrounding blaIMP-4 in these two plasmids were identical to IncN plasmids pMP-HK1500 (KT989599) and pP10159-2 (MF072962), both from *Citrobacter freundii*. The blaIMP-1 gene in pECC33-49 was situated within a Tn402-like integron, which was atypical due to a lack of 3′-CS. A similar genetic structure of blaIMP-1 (intI1-aacA7-DorF-blaIMP-1-ΔtniA) was also found...
Identification and characterization of an NDM-1- and IMP-1-coproducing ECC

The isolate ECC33, harboring two MBLs (NDM-1- and IMP-1) and conferring high-level resistance to meropenem (MIC of 256 mg/mL), was isolated from the urine of an old patient who was diagnosed with urinary tract infection in a tertiary hospital in Zhejiang, China. The ANI result revealed that ECC33 shared the highest identity (99.05%) with Enterobacter hormaechei subsp. hormaechei ATCC 49162 (AFHR00000000). MLST showed that ECC33 belonged to ST78, which was thought to be a high-risk clone among both ESBL-producing ECC and carbapenem-resistant Enterobacter cloacae complex (CREC) (16). The ECC33 genome consisted of a circular chromosome and two plasmids. The \(\text{bla}_{\text{IMP-1}}\) was carried on the IncP-1 \(b\) plasmid pECC33-49, which encoded 56 open reading frames (ORFs) with a length of 49,381 bp, and the \(\text{bla}_{\text{NDM-1}}\) was carried on the IncN plasmid pECC33-57, which was 57,389 bp in length and contained 72 ORFs.

pECC33-57 carried several antimicrobial resistance genes (\(\text{bla}_{\text{NDM-1}}, \text{ble}, \text{qnrS1}, \text{and dfrA14}\) conferring resistance to \(\beta\)-lactams, bleomycin, quinolones, and trimethoprim. Sequence analysis indicated that pECC33-57 also carried different mobile genetic elements (MGEs), including one class 1 integron In191 (Fig. 3). Comparative genomic analysis revealed that pECC33-57 shared the highest sequence similarities with four \(\text{bla}_{\text{NDM-1}}\)-carrying IncN
plasmids, namely, pNDM1-CBG (CP046118.1; 97% coverage and 100% identity), pSCH6109-NDM (CP050859.2; 97% coverage and 99.95% identity), pNDM1_LL34 (CP025965.2; 97% coverage and 99.99% identity) and pNDM-BTR (KF534788.2; 98% coverage and 99.99% identity), especially true in the classical and highly conserved backbones of these plasmids (Fig. 3). Interestingly, compared with these four plasmids, one extra IS30 inserted upstream of In191 was observed in pECC33-57, which may be associated with the mobilization of In191.

Only one antimicrobial resistance gene, blaIMP-1, located within a Tn402-like integron, was found in pECC33-49. An operon related to resistance to mercury (merEDAPTR) was also found in pECC33-49. Moreover, plasmid pECC33-49 contained two transfer-related regions, one consisting of 15 tra genes (traC to traO) and the other consisting of 16 trb genes (trbA to trbP) (Fig. 4). Notably, when searching for pECC33-49-like genomes (>80% coverage and >80 identity) in the NCBI database, none of the related genomes identified were from the Enterobacter cloacae complex. In contrast, we found four blaIMP-1-encoding IncP-1β plasmids from Achromobacter xylosoxidans (pA22732-IMP, 99.95% coverage and 99.98% identity; KJ588780.1), Morganella morganii (pNXM63-IMP, 98.76% coverage and 99.99% identity; MW150990.1), and Aeromonas caviae (pKAM345_1, 91.60% coverage and 99.93% identity; AP024949.1; pKAM339_2, 91.61% coverage and 99.93% identity; AP024942.1). Phylogenetic analysis of these five IncP-1β plasmids revealed that pECC33-49 has a close relationship with plasmid pNXM63-IMP (MW150990.1) from Morganella morganii (Fig. S5 in Supplemental File 1).
finding indicated that the pECC33-49-like plasmids were more likely to be transferred between bacterial species of different genera.

**DISCUSSION**

ECC are common nosocomial pathogens ranking as the top three *Enterobacteriaceae* in healthcare-associated infections (3), which has attracted wide public attention. Nevertheless, the presence and emergence of multiple species/subspecies of ECC have led to diversity and complications at the taxonomic level. Moreover, it impedes the further understanding of the epidemiology and clinical significance of species/subspecies of ECC. Accurate identification of ECC species is extremely important. Previous studies have mainly classified ECC species by phenotype or *hsp60* typing (2). With the rapid development of sequencing technology, genotypic classification methods, such as ANI, have higher accuracy and resolution. In this work, 172 ECC clinical isolates were collected from three hospitals in different cities in Zhejiang Province in southern China, and then further classified 160 into 9 species based on ANI analysis.

*E. hormaechei* (45.14%) and *E. kobei* (13.71%) were the predominant species, and the high prevalence of *E. hormaechei* and *E. kobei* among ECC isolates was consistent with the findings of a previous study that investigated the characterization of ESBL-positive community-acquired ECC isolates from 31 hospitals in 12 provinces of China (6). Interestingly, because novel species recently described based on a computational analysis of sequenced *Enterobacter* genomes (4, 17), *E. bugandensis* and *E. roggenkampii* were also identified in this study, accounting for 7.56% and 8.72% of the isolates, respectively. However, due to the limited sequencing data available in public databases, 12 isolates without characterized species/subspecies references could only be clustered into 4 clades (L, O, P and T) according to the classification method described by Sutton et al. (4), and these isolates will be further analyzed and might be classified as novel species/subspecies.

One hundred and twenty-six STs, including 62 novel STs, were found among the 172 isolates, with ST78 (4.07%) and ST171 (3.49%) ranking first and second, respectively. This large number and a great variety of STs, especially with half of the STs in such a small population being novel, indicated the members of the ECC group might have been evolving in clinical settings. A similar phenomenon was observed in a multicenter study in which novel STs accounted for a significant proportion (87.50%) (18). ST78 and ST171 were the most prevalent STs among the isolates in this work. A previous study revealed that ST78 and ST171, as high-risk CREC clones, were widely distributed and had high epidemic potential (19). The potential drug-resistant outbreaks caused by these STs still need robust surveillance.

Almost all of the isolates (170/172; 98.86%) carried AmpC β-lactamase genes, with *bla*<sub>ACT</sub> genes being the most dominant (58.70%), indicating that inducible ACT AmpC enzymes are conserved among ECCs. Intriguingly, the ACT type β-lactamase genes exhibited a certain species specificity. For instance, *bla*<sub>ACT-2</sub> and *bla*<sub>ACT-3</sub> were only present in *E. asburiae*, whereas *bla*<sub>ACT-9</sub>, *bla*<sub>ACT-12</sub> and *bla*<sub>ACT-6</sub> exclusively occurred in *E. kobei*, *E. ludwigii*, and *E. mori*, respectively. DHA, the most prevalent plasmid-mediated AmpC β-lactamase that might confer slight resistance to carbapenem (20), was found to be less abundant in the *E. cloacae* complex. Interestingly, compared to the previous findings that the *bla*<sub>NDM-1</sub> gene was most prevalent among the carbapenem-resistant isolates found in northeastern (e.g., Liaoning Province) (21), southern (e.g., Guangdong Province), and northwestern (e.g., Ningxia) (22) regions, the CREC strains we found here were dominated by *bla*<sub>NDM-5</sub> (6/14; 42.86%), which might be due to differences in antibacterial agents use in different regions of China, or may be caused by the limited number of strains we collected. Moreover, a higher frequency of other acquired β-lactamase genes, such as *bla*<sub>TEA</sub>, was observed in *E. hormaechei* and *E. kobei* isolates, indicating that the strains of the two species are more likely to spread and survive in hospital settings. Pan-genome analysis of the two species revealed that they possessed a greater capacity to acquire exogenous genes (23), which might partly explain the higher frequency of resistance genes in these species.

Notably, a strain named ECC33 harboring two MBL genes, *bla*<sub>NDM-1</sub> and *bla*<sub>AMP-1</sub>, was...
identified in our study. To the best of our knowledge, although a previous study has reported an NDM-1 and IMP-1 co-expressing Enterobacter cloacae strain based on next-generation sequencing in Ningxia in northwest China (22), this is the first report of detailed characterization of NDM-1 and IMP-1 encoded on two plasmids, respectively, in an E. hormaechei isolate. MLST assigned this isolate to ST78, a high-risk clone among both ESBL-producing ECC and CREC (19). There is no doubt that the emergence of the ST78 clone harboring two MBLs increases the difficulty of clinical treatment. Interestingly, compared with the blaOXA-1 encoding plasmid pECC33-49 from ECC33, 4 similar plasmid sequences (from Achromobacter xylosoxidans, Morganella morgani, and Aeromonas caviae) with >91.0% coverage and >99.0% identity were found from genera other than the E. cloacae complex in the NCBI database. The diversity of the origins indicated that the pECC33-49-like plasmids are widely distributed in different species and may be captured by E. hormaechei through the high-frequency transfer of the recombinant plasmids.

MATERIALS AND METHODS

Clinical strain collection. A large number of isolates from patients with active disease were continuously collected from three districts (Wenzhou, Hangzhou, and Huzhou) in Zhejiang Province, China between 2019 and 2020. After genetic identification of isolates as strains of Enterobacter cloacae complex, a total of 172 clinical ECC isolates were obtained from three tertiary hospitals (100 strains from hospital A in Wenzhou, 64 strains from hospital B in Hangzhou, and 8 strains from hospital C in Huzhou) for further study.

Antimicrobial susceptibility test. Antimicrobial susceptibility was determined using the agar dilution method on Mueller-Hinton (MH) agar plates supplemented with different concentrations of antibiotics. The test was repeated three times to ensure accuracy. The MICs were then interpreted following the breakpoint criteria of the Clinical and Laboratory Standards Institute (CLSI) for Enterobacteriaceae (24). Escherichia coli ATCC 25922 was used as the MIC reference strain for quality control. Those isolates which conferred resistance to meropenem were considered to be carbapenem resistance phenotypes. ESBL confirmation test was also performed according to the method recommended by CLSI and positive strains were considered to be ESBL positive phenotype.

Whole-genome sequencing and sequence analysis. Genomic DNA was extracted from each isolate using an AxyPrep bacterial genomic DNA miniprep kit (Axygen Scientific, Union City, CA, USA). The library with an average insert size of 400 bp was prepared using NEBNext Ultra II DNA library preparation kit, and genomic sequencing by the Illumina HiSeq 2500 platform (paired-end run; 2 × 150 bp) was performed at Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China). Genomic sequencing was performed by the Illumina HiSeq 2500 platform. Sequence assembly was conducted de novo on Illumina short reads using SPAdes v.3.14.0 (25). The genomes of isolates carrying MBL genes were further sequenced by PacBio RS II instruments (Pacific Biosciences, CA, USA). The PacBio data were first assembled using Canu v2.1 (26) and then the assembled genomes were corrected using Illumina HiSeq data via Pilon v1.23 (27). Gene annotation was performed using the Prokka annotation pipeline (28) and corrected via BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Antimicrobial resistance genes were detected using ResFinder (29), and plasmid replicon types were identified using PlasmidFinder (30). MLST analysis was performed using the MLST database (http://pubmlst.org/ecloacae). The population evolutionary relationship of ST was analyzed using GrapeTree (https://enterobase.readthedocs.io/en/latest/index.html). Mobile genetic elements (MGEs) were identified using ISFinder (31) and INTEGRALL (32) with default parameters. Gene distribution was visualized using the ComplexHeatmap package in R (33). Core genes of E. cloacae complex genomes were constructed using kSNP (34) to call single nucleotide polymorphisms (SNPs) and the recombined regions within the core genome were detected using Gubbins v2.4.1 (35). The phylogenetic tree was visualized in ggtree package in R (36). Genetic context analysis was performed using BLASTN and visualized in genoplotR (37). The BLAST Ring Image Generator (BRIG) (38) tool was used to generate the circular maps of the plasmids pECC33-57 and pECC33-49.

Pangenome analysis and COG functional characterization. Analysis of the pangenomes of two species (E. hormaechei and E. kobei) was carried out using Roary v3.13.0 (39). The rarefaction curve of pan-genomes and core-genomes of selected species was visualized via ggplot2 (40). COG categorization of each pan-genome was performed using EggNOG-mapper v2.1.0 (41) with an E value < 1e−10, an identity higher than 40%, and a coverage higher than 70%.

Species identification using ANI. Due to the diversity of the subspecies of the Enterobacter cloacae complex, average nucleotide identity (ANI) analysis was performed for the isolates collected. The type strains used for the ANI analysis are listed in Table S1 in Supplemental File 1. The ANI analysis was computed using FastANI v1.31 (42), and a value >95% was used as the threshold for species definition.

Ethics approval. Individual patient data were not involved, and only anonymous clinical residual samples during routine hospital laboratory procedure were used in this study. It was approved by the ethics committee of Zhejiang Hospital, Zhejiang, China.

Data availability. The complete nucleotide sequences of the chromosome and two plasmids (pECC33-49 and pECC33-57) of ECC33 in this work have been submitted to the GenBank database under accession numbers CP098486, CP098487, and CP098488, respectively. The raw data of isolates collected in this study have also been submitted to the NCBI SRA database under the accession number PRJNA871306.
SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.1 MB.

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We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Q.B., C.C., and W.P. conceived and designed the experiments. M.Z., Y.L., D.H., L.W., F.D., J.L., X.L., and K.L. performed the experiments. X.D., C.Y., and L.Z. performed the data analysis and interpretation. X.D., M.Z., Q.B., C.C., and W.P. drafted the manuscript.

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