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Emergence of a novel Enterobacter kobei clone carrying chromosomally-encoded CTX-M-12 with diversified pathogenicity in northeast China

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Dear Sir,

Enterobacter cloacae complex (ECC), comprising at least five species (Enterobacter cloacae, Enterobacter kobei, Enterobacter asburiae, Enterobacter hormaechei and Enterobacter ludwigi) with different subspecies, has emerged as one of the important nosocomial pathogens in the last decade, responsible for 65%–75% of all infections due to Enterobacter species [1]. Recently, some potentially high-risk international clones causing nosocomial infections were revealed in a Europe-wide survey [2]. In this study, we characterized a CTX-M-12-producing ECC clone responsible for severe infections circulating in northeast China.

Three ECC strains were isolated from bronchoalveolar lavage (ECC3018), blood (ECC3026) and abscesses (ECC3047) of three patients admitted to a secondary hospital in a northeast province (Liaoning) in China. The isolates were further identified as E. kobei by hsp60 typing [3]. The three strains exhibited similar antimicrobial profiles determined by the agar dilution method (Table 1). They showed an extended spectrum β-lactamase-positive and AmpC-overexpression phenotype detected as previously reported [2]. Multilocus sequence typing with use of seven loci (ddnA, fuaA, gyrB, leuS, pyrG, rplB and rpoB) detected a new profile (3-3-110-32-19-16-17) for all isolates, and was assigned as ST591 by the PubMLST database (https://pubmlst.org/eco cloacae/).

Isolates were sequenced using Illumina HiSeq2500 (Illumina, San Diego, CA, USA) using 2 × 125 bp pair-end libraries. Genomes were assembled by CLC GENOMIC WORKBENCH v8.0, and annotated by the RAST service (http://rast.nmpdr.org/). To analyse the resistome, genomic sequences were uploaded to RESFINDER (https://cge.cbs.dtu.dk). The three isolates shared an identical resistome comprising 19 genes, including: aac(3)-IId, aac(6’)-II, aadA2, aadA16, aph(3’)-lc and armA for aminoglycoside resistance; blaCTX-M-12, blaCARB-2, blafaTEM-1 and an unnamed blaACT gene for β-lactam resistance; and other genes for various drug resistance (fmrA, mcr(E), mph(A), mph(E), ere(B), sul1, sul2, cmIA1, dfrA1). The genotypes can fully explain the results of susceptibility tests.

The blaCTX-M-12 is a rare CTX-M gene, and its genetic environment was identical in the three strains (Fig. 1a). An ISecp1 was located 48-bp upstream of blaCTX-M-12, and the structure was identical to that of Escherichia coli isolates (DQ658220) identified in Korea. The transposition unit ISecp1-blaCTX-M-12orf477 was highly similar to the well-reported typical blaCTX-M-15 transposition unit with a length of 2971 bp. The unit was integrated into the chromosome, located upstream of a pseudo gene and downstream of a gene encoding an asparaginyl-tRNA synthetase. Two 5-bp direct repeats TATTA were identified adjacent to the flanking left inverted repeat and putative right

TABLE 1. MICs of some antibiotic agents for Enterobacter kobei isolates

| Antibiotic     | ECC3018 | ECC3026 | ECC3047 |
|----------------|---------|---------|---------|
| Ampicillin     | >512    | >512    | >512    |
| Ampicillin-sulbactam | 64/32   | 32/16   | 32/16   |
| Ciprofloxacin  | 0.006   | 0.006   | 0.003   |
| Levofloxacin   | 0.03    | 0.03    | ≤0.015  |
| Fosfomycin     | 32      | 16      | 16      |
| Piperacillin   | 256     | 256     | 256     |
| Piperacillin-tazobactam | 8/4     | 8/4     | 4/4     |
| Amikacin       | >256    | >256    | >256    |
| Gentamicin     | 128     | 128     | 128     |
| Cefepime       | 4       | 8       | 8       |
| Cefoxime       | 4       | 4       | 4       |
| Cefoperazone-sulbactam | 64/32   | 64/32   | 32/16   |
| Ceftiraxone    | 32      | 16      | 32      |
| Ceftazidime    | 1       | 1       | 1       |
| Cefoxitin      | 128     | 128     | 128     |
| Cefazolin      | >128    | >128    | >128    |
| Ceftriaxone    | >128    | >128    | >128    |
| Meropenem      | ≤0.015  | ≤0.015  | 0.03    |
| Imipenem       | 0.25    | 0.25    | 0.25    |

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inverted repeat (Fig. 1a), suggesting that IS\textit{Ecp1} mediated the chromosomal integration of \textit{bla}CTX-M-12.

The \textit{bla}CTX-M-12 gene was first detected from a \textit{Klebsiella pneumoniae} outbreak clone in Kenya in 2001 [4], and was later identified in \textit{Escherichia coli} and \textit{K. pneumoniae} isolates from Colombia and Korea, respectively [5,6]. To our knowledge, this is the first report of \textit{bla}CTX-M-12 identified in \textit{E. kobei}. As the geographic area where our strains isolated is bounded on the south by Korea, it is possible that the emergence of \textit{bla}CTX-M-12 may be due to cross-border spread. Further surveillance for \textit{bla}CTX-M-12 should be carried out to test this hypothesis.

Additionally, among \textit{Enterobacteriaceae}, chromosome-encoded CTX-Ms are frequently found in \textit{Escherichia coli}, \textit{K. pneumoniae} and \textit{Proteus mirabilis}, but are very rare in ECC.

FIG. 1. Schematic diagram of genetic environment surrounding \textit{bla}CTX-M-12 and \textit{armA}. Genes are shown in red (resistance genes), green (genes of mobile genetic elements), yellow (inverted repeats) and blue (other genes) arrows. The annotation of hypothetical genes is not shown. (a) The cassette \textit{IS\textit{Ecp1}-bla}CTX-M-12-orf477 was integrated into the chromosome, and its insertion site is shown; (b) the \textit{armA} gene located in a Tn1518-like composite transposon.

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Additionally, this is the first evidence of chromosomal integration of \( \text{bla}_{\text{CTX-M-12}} \), which had previously been exclusively detected in plasmids. This suggests the role of chromosomal locations in the spread of \( \text{bla}_{\text{CTX-M-12}} \).

The three isolates showed high MIC values of amikacin (>256 mg/L). A 16S rRNA methylase gene \( \text{armA} \) was detected in a \( \text{Tn1548} \)-like segment in the isolates responsible for the amikacin resistance (Fig. 1b). This \( \text{Tn1548} \)-like segment consisted of a 3′-end conserved region and a 5′-end class 1 integron. The conserved region was structured as \( \text{ISCR1-ISEc28-armA-ISEc29-msr(E)-mph(E)} \), and the variable region of class 1 integron carried \( \text{aac(6')-II} \) and \( \text{aadA16} \). Notably, the region from an IS26-disrupted \( \text{intI1} \) gene to an IS\( \text{Ab24} \) was identical to that identified in \( \text{Acinetobacter baumannii} \) strain A071 (KT317079) (Fig. 1b). Both ends of the \( \text{Tn1548} \)-like segment were disrupted by IS26, indicating that IS26 mediated the mobilization of this composite transposon cross species. The concomitance of extended spectrum \( \beta\)-lactamases and 16S rRNA methylases raises clinical concern and may become a major therapeutic threat in the future.

The genetic diversity of the three strains was determined by single-nucleotide polymorphism (SNP) analysis as described previously [8], and they differed by 57 SNPs (see Supplementary material, Table S1). This excludes the possibility of a recent transmission among the three patients, suggesting a clonal dissemination in the region. To investigate whether the genetic differences were associated with alterations of biological function, biofilm formation was tested by microtitre plate assay as described previously [9]. Intriguingly, ECC3018 could form four- to ten-fold more biofilm (24 h 0.12 ± 0.02; 48 h 0.18 ± 0.04) than the other two (24 h 0.012 ± 0.003, 0.011 ± 0.002; 48 h 0.048 ± 0.03, 0.037 ± 0.02) (p <0.05) at 37°C. The discrepancy could be explained by multiple non-synonymous SNPs identified in the genes involved in biofilm formation (see Supplementary material, Table S1), including \( \text{barA} \) encoding a sensory histidine kinase [10], \( \text{kefA} \) encoding a potassium efflux system [11] and \( \text{malT} \) encoding a transcriptional activator of maltose regulon [12]. Additionally, more non-synonymous SNPs than synonymous ones (36 versus 12) were identified in the clone frequently associated with genes involved in metabolism, membrane and pathogenicity. This implicates that the clone underwent positive selections resulting in pathogenicity diversification.

In summary, this study raises the concern that a wide repertoire of resistance mechanism and enhanced pathogenicity detected in the novel \( \text{E. kobei} \) clone increases its epidemic potential, and highlights the necessity of surveillance on the potential high-risk clone in the future.
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