A Novel $\text{bla}_{\text{CTX-M-65}}$-Harboring IncHI2 Plasmid pE648CTX-M-65 Isolated from a Clinical Extensively-Drug-Resistant Escherichia coli ST648

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Background: An ESBL, carbapenemase- and MCR-1-producing Escherichia coli ST648 strain was isolated from the urine sample of a patient in a Chinese tertiary hospital in 2016.

Methods: The strain was fully sequenced by GridION X5 platform of Oxford Nanopore Technology.

Results: The sequence analysis showed that the extended-spectrum $\beta$-lactamases CTX-M-65 and OXA-1, the carbapenemase NDM-5, the MCR-1 were encoded, respectively, by three different resistance plasmids. The pE648CTX-M-65-carrying $\text{bla}_{\text{CTX-M-65}}$ was a novel conjugative plasmid belonging to IncHI2 type; except for the $\text{bla}_{\text{CTX-M-65}}$, it also carried resistance genes $\text{ble, floR, sul1, aph(4)-Ia, aac(3)-VI, aac(6)-II, bla}_{\text{OXA-3}}, \text{catB, arr3 and tetA}$. Besides, an IncX4 plasmid pE648MCR-1-carrying $\text{mcr-1}$ and an IncX3 plasmid pE648NDM-5-carrying $\text{bla}_{\text{NDM-5}}$ were also identified.

Conclusion: The three transferable resistance plasmids coexisting in the E. coli ST648 isolate indicated the high risk to disseminate the extensively-drug-resistance among Enterobacteriaceae.

Keywords: CTX-M-65, MCR-1, NDM-5, extensively-drug-resistance, Escherichia coli

Backgrounds

The global increase in carbapenemase-producing Enterobacteriaceae has resulted in increased use of colistin with the inevitable risk of emerging pan-drug-resistant Gram-negative bacteria. MCR-1-producing carbapenem-resistant Enterobacteriaceae (CRE) isolates pose a significant threat to global health. In 2016, two Escherichia coli isolates ST648 and ST156 coproducing MCR-1 and NDM-5 were first reported from a duck sample in China. Subsequently, the coexistence of MCR-1 resistance and the NDM-5 has been reported in E. coli and Klebsiella pneumoniae isolates cultured from patients in the USA and China; E. coli isolates, such as ST206, ST167, ST156 and ST405, harboring $\text{mcr-1}$ and $\text{bla}_{\text{NDM-5}}$ have been reported causing intra-abdominal, bloodstream and urinary tract infections of patients. So, Feng et al concluded that the dissemination of $\text{mcr-1}$ colistin resistance gene is ongoing by clonal expansion among different sequence types of E. coli. MCR-1-producing CRE isolates represent a great concern for public health.

In the reported E. coli ST648 isolate coproducing MCR-1 and NDM-5 cultured from a duck, the $\text{bla}_{\text{NDM-5}}$ was found on an IncX3 plasmid while the $\text{mcr-1}$ gene was located on an IncHI2 plasmid, besides, the isolate also harbored extended-spectrum $\beta$-lactamases CTX-M-65 and OXA-1.

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β-lactamases (ESBL) genes bla<sub>TEM-1</sub> and bla<sub>CTX-M-55</sub>, fosfomycin resistance gene fosA3 and quinolone resistance gene aac(6′)-Ib.<sup>2</sup> E. coli ST648 strain, a predominant multidrug-resistant clone observed worldwide in humans, companion animals, livestock, and wild birds, is frequently associated with various β-lactamases, including ESBLs, NDM and KPC.<sup>3-11</sup> So the epidemic multiresistant E. coli ST648 clone might raise a potential threat to human health.<sup>2</sup> The first clinical E. coli ST648 isolate with mcr-1 and bla<sub>CTX-M-15</sub> located on different plasmids was cultured from the microbiota of diarrhea patients in China in 2016, which NDM-1 variants NDM-5 or NDM-7 were not found in it.<sup>7,12</sup> Here we firstly reported the clinical E. coli ST648 isolate coharboring ESBL genes bla<sub>CTX-M-65</sub> and bla<sub>ROXA-1</sub>; the carbapenemase gene bla<sub>NDM-5</sub>; the colistin-resistant gene mcr-1 located, respectively, in three different resistance plasmids.

The bla<sub>CTX-M-9</sub>-group gene bla<sub>CTX-M-65</sub> has become one of the dominant CTX-M types in animal isolates in China since 2007,<sup>13,14</sup> then it has rapidly spread and become the most common ESBL type instead of bla<sub>TEM</sub> and bla<sub>SHV</sub> in the acquired infection of the hospital and the community in China.<sup>15,16</sup> The IncF1 plasmids were the main vectors to transmit the bla<sub>CTX-M-65</sub> among Enterobacteriaceae in China.<sup>17</sup> The bla<sub>CTX-M-65</sub> genes often coexist with genes encoding 16S rRNA methylases such as armA and rmtB, or with fosA3 that confers resistance to fosfomycin.<sup>17-19</sup> The IS<sup>Ecp1</sup>-bla<sub>CTX-M-65</sub>-IS903-iroN structure is a typical transposition unit in which bla<sub>CTX-M-65</sub> locates.<sup>17</sup> But a novel conjugative bla<sub>CTX-M-65</sub>-carrying plasmid designated pE648CTX-M-65 (241,735bp) belonging to IncH1 type was firstly reported here, which the bla<sub>CTX-M-65</sub> located in a different mosaic structure. Besides, a mcr-1-carrying plasmid designated pE648MCR-1 (33,277bp) and a bla<sub>NDM-5</sub>-carrying plasmid designated pE648NDM-5 (47,827bp) were also identified. This represents the clinical clone of E. coli ST648 can spread extensively-drug-resistance among Enterobacteriaceae.

**Materials and Methods**

**Bacterial Isolates and Identification**

The isolate was cultured from the urine specimen of a 55-year-old male patient suffered from recurrent urinary tract infection, along with bladder cancer, hypertension and coronary disease in a tertiary hospital of Xuzhou, China, in 2016. The patient had not been travelling and not engaged in animal breeding. The urinary tract infection was treated by ceftazidime and levofloxacin at the beginning. The bacterial species identification was performed using BioMérieux Vitek 2, Bruker MALDI Biotyper and 16S rRNA gene sequencing. The sequence type was identified by using the genome sequence to query the PubMLST database using the software written by Dr Torsten Seemann.<sup>20</sup> The seven house-keeping genes (adh, fumC, gyrB, icd, mdh, purA, recA) were assigned an allele number and a sequence type (ST) was determined for the isolate according to the allele profile. The phylogenetic tree of the five E. coli ST648 strains available as full genome sequences in Genbank was inferred using maximum likelihood as part of the software kSNP3 on all complete genomes.<sup>21</sup>

**Antimicrobial Resistance**

The minimum inhibitory concentrations (MICs) of antimicrobial agents were determined using the microdilution broth method according to the 2020 CLSI guidelines. The antimicrobial susceptibility profiles were interpreted by the 2020 CLSI guidelines and EUCAST available version (http://www.eucast.org/). The ESBL genes, carbapenemase genes and the mcr-1 gene were screened for by PCR with specific primers,<sup>1,22</sup> as shown in Supplementary materials Table S1.

**Plasmid Transfer**

Conjugal transfer experiment was performed with *Escherichia coli* J53 Azi<sup>®</sup> as the recipient strain. Overnight cultures of the bacteria were diluted to 1.5×10<sup>8</sup> cells/mL. Donor and recipient cells were mixed at 1:10 donor-to-recipient ratio, after 18 h of incubation of donor-recipient mixtures on blood plates at 35°C, cells were washed by normal saline solution and diluted to be cultured selectively on MacConkey agar plates supplemented with sodium azide (100 μg/mL) and ceftriaxone (10 μg/mL), or imipenem (1 μg/mL), or colistin (2 μg/mL) for 24 h. Transconjugants were confirmed by PCR amplifying bla<sub>CTX-M-9</sub>-group, bla<sub>NDM</sub> and mcr-1 with corresponding primers as shown in Table S1.

**Whole Genome Sequencing and Data Analysis**

Genomic DNA of the *E. coli* isolate was extracted using a QIAGEN Genomic-tip 500/G (Cat No.10262, QIAGEN, Duesseldorf, Germany). We applied an Oxford Nanopore...
Technology’s (ONT) GridION X5 sequencing technology for the sequencing.\textsuperscript{23,24} The strategy yielded a total of 4724.41 Mb raw data and a total of 4723.08 Mb filtered data were finally obtained after removing reads with mean_qscore <7 and length <1000 bp. The filtered reads were \textit{de novo} assembled using the Canu package (version 1.3) with default parameters and the assembled data were fixed by Pilon (version 1.22) with default parameters, using the data obtained from the Illumina sequencing performed on an Illumina HiseqX10 platform (IlluminaInc., SanDiego, CA, USA) as the reference. The finally resulting genomes including the single circular chromosome and the circular plasmids were annotated using the best-placed reference protein set (GeneMarkS+) in the NCBI Prokaryotic Genome Annotation Pipeline (version 3.3) and the RAST tool (version 2).\textsuperscript{25–27} The complete genome and plasmids sequences of the \textit{E. coli} isolate 201,609 were submitted to GenBank under accession number CP048107 (Chromosomal genome sequence), MN200941 (Plasmid sequence of pE648CTX-M-65), MN200942 (Plasmid sequence of pE648NDM-5) and MN200943 (Plasmid sequence of pE648MCR-1).

\section{Results and Discussion}

\subsection*{Antibiotic Resistance of the \textit{E. coli} Isolate}

The isolated \textit{E. coli} 201,609 was resistant to piperacillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/tazobactam, cefoxitin, ceftazidime, cefotaxime, cefepime, aztreonam, imipenem, meropenem, ertapenem, amikacin, gentamycin, ciprofloxacin, levofloxacin, sulfoxmethoxazole, tetracycline and colistin, but susceptible to fosfomycin and tigecycline; see \textbf{Table 1}. We screened for the resistance genes by PCR as mentioned in Materials and Methods, the \textit{bla}_{CTX-M-9}-\textit{group}, \textit{bla}_{GXXA-1}, \textit{bla}_{NDM} and \textit{mcr-1} genes were successfully amplified. In the conjugation experiment, we also detected the \textit{bla}_{CTX-M-9}, \textit{bla}_{NDM} and \textit{mcr-1} genes, respectively, by PCR in the recipient \textit{E. coli} 353 Azi\textsuperscript{7}, indicating resistance plasmids were transferable.

\subsection*{Genome Sequence of the \textit{E. coli} Isolate}

The complete genome of the \textit{E. coli} isolate 201,609 was 5.53Mb which contained a 5.20Mb circular chromosome and three resistance plasmids named pE648CTX-M-65, pE648NDM-5 and pE648MCR-1, respectively. The GC percentage of the genome is 50.2%. The average depth of the chromosome (the raw data/the assembled data; per reference start position) was 175X for Nanopore. The coverage of the circular chromosome (length alignment to plasmid_ databank/the whole length) was 19.58%, while the coverage of the three resistance plasmids was over 95%. The sequence type was identified as ST648 according to the allelic profile of the seven house-keeping genes (\textit{adh}, \textit{fumC}, \textit{gyrB}, \textit{icd}, \textit{mdh}, \textit{purA}, \textit{recA}) which were assigned an allele number (92, 4, 87, 96, 70, 58, 2). The relatedness of the isolate in this study (Genbank ID: CP048107) to another four multiple antibiotic-resistant \textit{E. coli} ST648 strains whose genomes were available in Genbank was shown in \textbf{Figure 1}. The four resistance strains were, respectively, isolated from a hospital of Beijing in 2009 (Genbank ID: CP008697), a hospital of Greek in 2018 (Genbank ID: CP035318 and CP035317) and a wild bird of Germany in 2012 (Genbank ID: CP023815). The phylogenetic tree generated using kSNP3.0 indicated the isolate in this study had a more close evolutionary distance to the one isolated from a wild bird compared with the another three isolates from hospitals.\textsuperscript{28}

\begin{table}
\centering
\caption{Antimicrobial Drug Susceptibility Profiles}
\begin{tabular}{|c|c|}
\hline
\textbf{Antibiotics} & \textbf{MIC (mg/L)} \\
\hline
\textit{E. coli} ST648 & \\
\hline
Piperacillin & \geq 128 \\
Amoxicillin/clavulanic acid & \geq 32 \\
Ampicillin/sulbactam & \geq 32 \\
Piperacillin/tazobactam & \geq 128 \\
Cefoxitin & \geq 256 \\
Ceftazidime & \geq 256 \\
Cefotaxime & \geq 256 \\
Cefepime & \geq 64 \\
Aztreonam & \geq 64 \\
Imipenem & \geq 16 \\
Meropenem & \geq 16 \\
Ertapenem & \geq 32 \\
Amikacin & \geq 64 \\
Gentamycin & \geq 16 \\
Ciprofloxacin & \geq 4 \\
Levofloxacin & \geq 8 \\
Sulfamethoxazole & \geq 320 \\
Tetracycline & \geq 16 \\
Colistin & \geq 8 \\
Fosfomycin & 1 \\
Tigecycline & 2 \\
\hline
\end{tabular}
\end{table}
Overview and Comparative Analysis of pE648NDM-5 and pE648MCR-1

The pE648NDM-5-carrying blaNDM-5 is a 47,827 bp circular plasmid containing 73 putative ORFs (35 hypothetical proteins), the average GC content is 47%. It is classified into IncX3 type according to PlasmidFinder. The direct spread of NDM-1 or NDM-5 by IncX3-type plasmids among Enterobacteriaceae was very common in China. Here, the pE648NDM-5 has 96% identity with the other two plasmids, the pNDM5_IncX3 (GenBank ID KU761328) isolated from a clinical K. pneumoniae and the pP855-NDM5 (GenBank ID MF547508) in an E. coli ST7511 strain isolated from a pig, as shown in Figure 4; indicating the epidemicity of blaNDM-5-carrying IncX3 plasmids among Enterobacteriaceae.

The mcr-1-carrying pE648MCR-1 is a 33,277 bp circular plasmid containing 54 putative ORFs (20 hypothetical proteins), the average GC content is 41.8%. It is classified into IncX4 type, which was also found very epidemic among mcr-1-carrying Enterobacteriaceae. Genome alignments indicated the pE648MCR-1 reported here was nearly identical to the pMCR1_IncX4 (GenBank ID KU761327) isolated from a clinical K. pneumoniae and the pGD65-4 (GenBank ID KY075660) in an E. coli isolated from swine, as shown in Figure 5. Besides, very similar plasmids (>95% identity) had been extensively reported in Salmonella enterica, K. pneumoniae and E. coli.
Figure 2 Sequence alignment of the pGD80-2 with the pE648CTX-M-65 generated by the Brig software.

Figure 3 Organization and comparison of pE648CTX-M-65 and pGD80-2. (B) Linear sequence comparison of pE648CTX-M-65 with pGD80-2. Regions of >99% identity are marked by light blue shading. (A) The MRR of pE648CTX-M-65. Genes are denoted by arrows and are colored based on gene function classification. Red, drug-resistance genes; green, mobile elements; violet, common genes; grey domain marked, the transposon.
Here, the \textit{mcr-1} and \textit{bla}_{\text{NDM-5}} were located on different transferable plasmids, while a IncX3-X4 hybrid pCQ02-121 (Genbank ID KU647721) coharboring the \textit{mcr-1} and \textit{bla}_{\text{NDM-5}} in an \textit{E. coli} ST156 strain was isolated from a Chinese pet cat in 2015. The author proposed that the stable hybrid pCQ02-121 origin from
the pNDM5_IncX3 (GenBank ID KU761328) and pMCR1_IncX4 (GenBank ID KU761327) isolated from a clinical K. pneumoniae ST25 strain in China by two IS26-mediated recombinations. Here, the sequence analysis of the pE648NDM-5 and the pE648MCR-1 which were highly homologous to the pNDM5_IncX3 and the pMCR1_IncX4, respectively, shown in Figures 4 and 5, was a more direct proof to support the recombination conclusions on the origin of a IncX3-X4 hybrid pCQ02-121, as shown in Figure 6. The majority (nt

**Figure 6** Sequence comparison of pE648NDM-5 and pE648MCR-1 with pCQ02-121. (A) Sequence Alignments generated by the Brig software. (B) Linear Comparison of the three plasmids with annotated genes. Genes are denoted by arrows and are colored based on gene function classification. Red, drug-resistance genes; green, mobile elements; violet, common genes. Regions of > 98% identity are marked by light blue shading.
8203–42968) of pCQ02-121 was almost identical to the pE648NDM-5 (nt 6389–41126) except for 4 mismatches and 22 gaps; while the region spanning nt 42958–9026 matched to the pE648MCR-1 (nt 27885–8994), only with 4 mismatches and 2 gaps.

Conclusions

The coexistence of ESBL, carbapenemase and MCR-1 leads to the dissemination of the extensively-drug-resistance among Enterobacteriaceae, which will inevitably become global. The finding of the extensively-drug-resistant *E. coli* ST648 201,609 strain stresses the need to monitor the use of colistin in treatment of both human beings and animals, as well as the need to surveil and restrict its further dissemination.

Ethical Approval

The urine specimen was part of the routine hospital laboratory procedure. The use of human specimens and all related experimental protocols was approved by the Committee on Human Research of the indicated institutions, and was carried out in accordance with the approved guidelines.

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Disclosure

The authors report no conflicts of interest in this work.

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