Comparative Genomic Characterization of Multidrug-resistant Citrobacter Spp. Strains in Fennec Fox Imported to China

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Abstract

**Background:** To investigate the antimicrobial profiles and genomic characteristics of MDR-*Citrobacter* spp. strains isolated from Fennec fox imported from Sudan to China.

**Methods:** *Citrobacter* spp. strains were isolated from stool samples. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) was used for identification. Antimicrobial susceptibility testing was performed using the broth microdilution method. Whole-genome sequencing was performed on an Illumina Novaseq-6000 platform. Acquired antimicrobial resistance genes and plasmid replicons were detected using ResFinder 4.1 and PlasmidFinder 1.3, respectively. Comparative genomic analysis of 277 *Citrobacter* genomes was also investigated.

**Results:** Isolate FF141 was identified as *Citrobacter cronae*, isolate FF371, isolate FF414, and isolate FF423 were identified as *Citrobacter braakii*. Of these, three *C. braakii* isolates were further confirmed to be ESBL-producer. All isolates are all MDR with resistance to multiple antimicrobials. Plasmids of incompatibility (Inc) group pKPC-CAV1321. Comparative genomics analysis of *Citrobacter* isolates generated a large core-genome. Genetic diversity was observed in our bacterial collection, which clustered into five main clades. Human, environmental and animal *Citrobacter* isolates were distributed into five clusters.

**Conclusions:** To our knowledge, this is the first investigation of MDR-*Citrobacter* from Fennec Fox. Our phenotypic and genomic data further underscore the threat of increased ESBL prevalence in wildlife and emphasize that increased effort should be committed to monitoring the potentially rapid dissemination of ESBL-producers with one health perspective.

Background

The worldwide increase and spread of infections caused by multidrug-resistant (MDR) Gram-negative bacteria of human and animal origin is a significant global public health burden in recent decades.[1, 2] Enterobacteriaceae are common bacteria and usually associated with different types of community-, hospital-acquired, and even animal infections,[3–6] so antimicrobial resistance (AMR) in these bacteria has significant potential impacts on control of AMR, with one health perspective.[7, 8] Among these organisms, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae are recognized as the most prevalent group of pathogens due to their mobility.[9, 10] Treatment of infections caused by ESBL, AmpC-, and carbapenemase-producing-producing Enterobacteriaceae strains is challenging, with limited evidence of their efficacy,[11] due to the emergence and spread of carbapenem resistance in ESBL-producing Enterobacteriaceae isolates, which is of particular clinical relevance.[12, 13]

The ESBLs are usually carried by mobile genetic elements, such as a variety of self-transferring plasmids, which can be transferred to other bacteria.[14–16] Thus far, several ESBL types have been identified in Enterobacteriaceae isolates of which the CTX-M, TEM, and SHV β-lactamases are the most prevalent groups.[17, 18] It is noteworthy that ESBL- and carbapenemase-producing Enterobacteriaceae occurring in animals has become a public-health issue in recent years.[4, 18] Zoonotic pathogens contributed to the cross-transmissions of ESBL-producing Enterobacteriaceae.[19] Previously, we also reported the detection of the transmission of AMR across human, animals and environmental compartments in China.[8, 20, 21]

*Citrobacter* spp. are common Gram-negative bacilli and widely found in water, food, soil, and intestines of animals and humans.[22] AMR encoding genes are frequently reported in *Citrobacter freundii*, and it became a reservoir of antibiotic resistance genes in recent years.[23, 24] In addition, MDR *C. freundii* has been reported in numerous hosts including but are not limited to humans.[25–27] In this work, we identified four MDR *Citrobacter* isolates in Fennec fox imported from Sudan to China. Antimicrobial susceptibility tests, conjugation experiments, whole-genome sequencing, and comparative genomic analysis were performed to study the molecular characteristics of these MDR strains.

Methods And Materials

**Bacterial identification and isolation of *Citrobacter* strains**

We collected 168 stool samples of wild Fennec fox imported from North Africa to China.[5] Stool samples were cultured by MacConkey agar supplemented with 1mg/L cefotaxime as described previously.[5] Bacterial identification was conducted by MALDI-TOF MS (Bruker, Bremen, Germany) as described.[16] Confirmation of ESBL-produing isolations was further performed by a standard double-disk diffusion method as defined by the Clinical and Laboratory Standards Institute (CLSI) ([https://clsi.org/](https://clsi.org/)).
Antimicrobial susceptibility testing (AST)

Susceptibility to 16 antibiotics (amikacin, aztreonam, cefpirome, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, florfenicol, fosfomycin, gentamicin, imipenem, meropenem, piperacillin-tazobactam, polymyxin E, tigecycline, tobramycin, and trimethoprim-sulfamethoxazole) for four MDR *Citrobacter* isolates were evaluated. The MICs were determined via an agar dilution method for all antibiotics except for colistin and tigecycline, for which a broth microdilution method was used according to the CLSI standards. *E. coli* ATCC 25922 was used as control.

Whole-genome sequencing (WGS) and bioinformatics analysis

Genomic DNA was extracted from four MDR *Citrobacter* isolates using the Qiagen Blood/Tissue kit (Qiagen, Hilden, Germany).[6] The sequencing library was prepared by using Illumina Nextera XT kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina NovaSeq 6000-PE150 platform (Illumina). Paired reads were then assembled into scaffolds using Velvet version 1.2.10.[43] Acquired antimicrobial resistance genes and plasmid replicons were performed using the CGE server (http://www.genomicepidemiology.org). Antibiotics Resistance Genes (ARGs) were identified using the ResFinder 4.1 database.[44] Genotyping was performed to query the seven domesticated genes (*aspC, clpX, fadD, mdh, arcA, dnaG, and lysP*) via the MLST database (https://pubmlst.org/organisms/citrobacter-spp). We further created a core genome-based phylogenetic tree using 4 *Citrobacter* genomes sequenced in this study and 272 randomly selected publicly available *Citrobacter* genomes (Table S1). The isolate collection includes strains from clinical (*n* = 159) and the environment (*n* = 133) sources that were widely distributed over time and geographical locations. *Citrobacter* genomes were annotated using Prokka[45] and RAST tool.[46] The core genes were identified using Prokka[45] and maximum likelihood-based phylogenetic reconstruction was performed with Roary.[47] Phylogenetic tree visualizations were generated by using iTOL (https://itol.embl.de/).

Plasmid characterization

The transferability of plasmids carrying MDR encoding genes was determined by filter mating as described previously.[20] Animal isolates and *Escherichia coli* J53 were used as donors and acceptors, respectively. The Animal isolates and J53 strains were mixed in (LB) broth at a ratio of 1:3 and incubated at 37°C for 18h. Transduction and binding were selected on MacConkey agar plates containing cefotaxime (2 μg/ml) and sodium azide (150 μg/ml) for 12h. Susceptibility test was performed to determine the horizontal transferability of drug resistance, and the corresponding transduction conjugate was confirmed by PCR amplification and pulsed field gel electrophoresis (PFGE).

Data availability

The whole-genome sequences of four *Citrobacter* spp. isolates have been deposited in the GenBank under the BioProject number PRJNA656097.

Results

Isolation and identification

In this work, four *Citrobacter* spp. isolates resistant to cephalosporins were cultivated by selective medium plates. Among these strains, isolate FF141 was identified as *Citrobacter cronae*, isolate FF371, isolate FF414, and isolate FF423 were identified as *Citrobacter braakii*. Of these, three *C. braakii* isolates were further confirmed to be ESBL-producer (Table 1).
Enterobacteriaceae in wild animals. coli reported to associate with nosocomial infections for a high mortality rate. spp. isolates represent up to 6% of all isolated Enterobacteriaceae from clinical specimens. The wide dissemination of MDR Enterobacteriaceae is a global concern with one health perspective.

Discussion

Assessment of antibiotic susceptibility and MLST analysis

Four Citrobacter isolates are all MDR with resistance to multiple antimicrobials (Table 1). The total resistance rate was observed for cefotaxime, ceftazidime, aztreonam, and chloramphenicol (100%). All the isolates were susceptibility to Imipenem, meropenem, amikacin, ticyceline, and colistin. Interestingly, only FF371 was resistant to piperacillin-tazobactam. Among these Citrobacter isolates, we found two sequence types (STs), which were ST350 (FF371, FF414, and FF423) and ST370 (FF141).

Resistant and virulence determinants of Citrobacter isolates

The acquired resistance genes detected in four Citrobacter isolates are summarized in Fig. 1A. The following genes were identified in three C. braakii isolates: the phenicol resistance gene floR; the trimethoprim resistance gene dfrA17; the tetracycline resistance gene tet(A); the disinfectant resistance gene qaeE; the quinolone resistance gene qnrS1; the fluoroquinolone and aminoglycoside resistance gene aac(6')-Ib-cr; the aminoglycoside resistance genes aph(6')-Id, aac(6')-Ib3, aph(3')-lb, aac(3)-Ild, and aadA5; the macrolide resistance gene mmpH; the ß-lactam resistance genes blaCTX-M-55, blaCMY-82, and blaoXA-1-; the rifampicin resistance gene ARR-3; sulphonamide resistance genes sul1, and sul2. Of note, C. braakii FF414 and FF423 encoded the fosfomycin resistance gene fosA3, while C. cronae FF141 and C. braakii FF371 were negative. Among four isolates, FF141 encoded fewer resistance genes than other isolates, which carried blaCMY-96, qnrB34, tet(A), dfrA12, and aadA2. Plasmids of incompatibility (Inc) group pKPC-CAV1321, was identified in FF141, and IncR was detected in other Citrobacter braakii isolates. The antimicrobial genes dfrA12, tet(A), and aadA2 genes were co-harborred by IncR plasmid in three C. braakii isolates.

Virulence gene analysis showed that the presence of genes encoding efflux pump protein (arcB), the transport of siderophores (fepC), enterobactin (entB and entE), outer membrane protein A (ompA), extracellular nucleation factors (csgB, csgD, csgE, and csgF), and type VI secretion system-related proteins (hcp/tssD) in all isolates (Fig. 1B). Moreover, three C. braakii isolates also carried genes that involved polymer synthesis (tviB-tviE), and cell surface localization of the CPS (vexA-vexE).

Comparative genomics analysis of Citrobacter isolates

Roary matrix-based gene sequence analysis generated 60,923 total genes and a large core-genome of 1,578 gene clusters of 277 whole genomes. The whole-genome phylogeny (Fig. 2) revealed a population structure. Genetic diversity was observed in our bacterial collection, which clustered into five main clades. Interestingly, C. braakii isolates have close relatedness with a clinical isolate from Viet Nam and an environmental strain, while C. cronae FF141 showed a high similarity with a clinical isolate from Nigeria and a clinical isolate from India (Fig. 2 & Table S1).

Discussion

The wide dissemination of MDR Enterobacteriaceae is a global concern with one health perspective.[28] In clinical settings, Citrobacter spp. isolates represent up to 6% of all isolated Enterobacteriaceae from clinical specimens.[29] Members of the genus Citrobacter are reported to associate with nosocomial infections for a high mortality rate.[30] The Citrobacter genus is most closely related to Escherichia coli and Salmonella, and is divided into 11 different genomospecies.[31] There is some information indicating a high prevalence of MDR Enterobacteriaceae in wild animals.[32, 33] Although previous studies suggest that fur animals are potential reservoirs of AMR, little

| Isolate | TZP | CTX | CAZ | CPO | IPM | MEM | AMK | ATM | CIP | CHL | FLR | GEN | TOB | FOS | TGC | COL |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| FF141   | 64  | 32  | 64  | 1   | 0.25| 0.0075 | 8   | 64  | 0.25| 64  | 16  | 4   | 0.25| 0.25| 2   |
| FF371   | 128 | > 128 | 64  | 128 | 0.25| 0.003 | 32  | 128 | 16  | 128 | > 128 | 64 | 256 | 0.25| 2   |
| FF414   | 2   | > 128 | 64  | 128 | 0.25| 0.003 | 16  | 128 | 16  | 128 | > 128 | 64 | 256 | 0.25| 2   |
| FF423   | 2   | > 128 | 64  | 128 | 0.25| 0.003 | 16  | 128 | 16  | 128 | > 128 | 64 | 256 | 0.25| 2   |

TZP: Piperacillin/Tazobactam; CTX: Cefotaxime; CAZ: Ceftazidime; CPO: Cefpirome; IMP: Imipenem; MEM: Meropenem; AMK: Amikacin; ATM: Aztreonam; CIP: Ciprofloxacin; CHL: Chloramphenicol; FLR: Florfenicol; GEN: Gentamicin; TOB: Tobramycin; FOS: Fosfomycin; TGC: Tigecycline; COL: Colistin
is known about the antimicrobial patterns, and genomic characteristics of MDR Citrobacter isolates from wildlife. In the present study, we first described the isolation of MDR-Citrobacter strains from Fennec Fox. We subsequently obtained the antimicrobial resistance profiles and genomic information by AST and WGS. We also identified antimicrobial resistance and virulence-associated genes.

Citrobacter spp. isolates can have chromosomal AmpC β-lactamases, as well as plasmid encoded carbapenemases, which results in ineffective of many antimicrobial agents for treatment.[21, 23, 35, 36] C. cronae was identified as a new Citrobacter species from human stool samples very recently,[29] the antimicrobial profiles of C. cronae are largely unknow. In this study, we first identified an AmpC β-lactamase encoding gene (bla<sub>CMY-98</sub>) in C. cronae, which provides a glimpse of antimicrobial insight into this species. Occurrence of ESBL-producing C. braakii isolated from animals and food product are occasionally reported.[37] Our detection of three ESBL-producing C. braakii isolates from Fennec Fox further suggests the risk of zoonotic potential MDR C. braakii from animals and animal products deservedly garners considerable attention.

Previous investigations found that CTX-M-55 became one of the prevalent ESBL type detected among clinical, animals, and environment in some countries.[14, 15, 38, 39] Very recently, the occurrences of CTX-M-55-producing Escherichia coli were also increasing reported in environment and diverse animal species in Europe.[40, 41] Our previous work confirmed that CTX-M-55-producing Escherichia coli was the most prevalent ESBL-producer from Fennec Fox.[5] These findings further strengthened that wildlife may act as potential reservoirs and vectors of CTX-M-55, although some of these isolates carried bla<sub>CTX-M-55</sub> genes on the chromosome.

Interestingly, the diverging clonality of the human, environmental and animal Citrobacter isolates was confirmed by the fact that strains originating in these three sources distributed into five clusters. It is still not sure whether the ecological and animal strains are highly related to the human strains in terms of genetic phylogeny. The previous investigation highlighted the challenges associated with species designation of Citrobacter by core genome analysis, particularly in regards to Citrobacter freundii, which did not constitute a discrete phylogenetic group.[42] As we noted in our data, C. cronae and C. braakii strains were clustered into the same clade, which suggests further accurate taxonomic inquiry is needed to clarify the lineage of Citrobacter members.

**Conclusion**

In summary, this investigation involved the first survey of MDR Citrobacter isolates in Fennec Fox. We characterized the phenotypic characteristics and genomic basis of MDR Citrobacter strains. Fennec Fox may serve as a common vector for the rapid dissemination of ESBL-encoding genes via animal contact and thereby threaten public health. Our findings further underscore the threat of increased ESBL prevalence in Enterobacteriaceae, and improved multisectoral surveillance for ESBL-producing Citrobacter is warranted.

**Declarations**

**Ethical approval** Not required.

**Consent for publication** Yes.

**Competing interests** None declared.

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**Author Contributions:** Conceptualization, Jie Qin.; methodology, Yishu Zhao.; software, Xiaohui Chi.; validation, Jie Qin., Yishu Zhao.; formal analysis, Xiaohui Chi.; investigation, Aifang Wang.; resources, Peipei Wen.; data curation, Shuang Li.; writing—original draft preparation, Hao Xu.; writing—review and editing, Sheng Bi.; visualization, Hao Xu.; supervision, Lingjiao Wu.; project administration, Hao Xu.; funding acquisition, Sheng Bi. All authors have read and agreed to the published version of the manuscript.

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**Figures**

A). Antimicrobial resistance gene profiles of 4 MDR-Citrobacter isolates. The heatmap is used to display the types of acquired AMR genes. Brown indicates the presence of AMR genes, whereas colorless correspond to the absence of the AMR genes.

B). The heatmap is used to display the types of virulence-associated genes. Brown indicates the presence of virulence genes, whereas colorless correspond to the absence of the virulence genes.

Figure 1
Figure 2

A core-genome analysis of 277 Citrobacter strains, including 4 isolates from this study and 273 strains downloaded from NCBI genome database. The year of the isolation is labelled in the outer ring. The source of the strains is presented in the middle ring. The location of the isolates is colored in the inner ring. Isolates identified in this study were colored in red. NA, details regarding the region of the strains is not available. The bar shows 100000 nucleotide substitution per position.

Supplementary Files

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