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Detection and characterization of ESBL-producing *Escherichia coli* expressing *mcr-1* from dairy cows in China

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Objectives: To investigate the prevalence and molecular characteristics of ESBL-producing *Escherichia coli* (ESBL-EC) in faecal samples from dairy cows in China.

Methods: In total, 651 faecal samples were collected from cows distributed among the 10 provinces of China. Potential ESBL-EC isolates were cultured on selective medium. The clonal relatedness of the ESBL-EC isolates was assessed using MLST. WGS was conducted on 3 *mcr*-positive isolates and 14 additional randomly selected ESBL-EC isolates. Southern blot, S1-PFGE and conjugation were performed for *mcr-1*-carrying isolates. The genetic environment of the pMCR-JLF4 plasmid was also analysed.

Results: In total, 290 unique ESBL-EC isolates were detected from 284 cows (43.6%). Alleles of CTX-M were observed in 94.1% (273/290) of all isolates. The most prevalent genotypes observed in this study were $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-17}}$ and $bla_{\text{CTX-M-55}}$. Differentiation of 79 STs with a polyclonal structure was accomplished using MLST. Clonal complex 10 was the most prevalent major complex detected here. Furthermore, the *mcr-1* gene was detected in three isolates. The complete sequence of the *mcr-1*-containing pMCR-JLF4 was determined. The plasmid was 66.7 kb in length, with a genetic structure of *nikA–nikB–mcr-1–pap2*. Conjugation analysis confirmed that the *mcr-1* gene in pMCR-JLF4 was transferable without the assistance of the IS*Apl1* gene.

Conclusions: The data presented here suggest high prevalence of ESBL-EC in Chinese cow farms. Furthermore, it was clearly demonstrated that commensal *E. coli* strains can be reservoirs of $bla_{\text{CTX-M}}$ genes, potentially contributing to the dissemination and transfer of the *mcr-1* gene to pathogenic bacteria among cows.

Introduction

Bacteria producing ESBLs have been implicated in cases of nosocomial, community-acquired and foodborne infections. Commensal isolates of *Escherichia coli* in food animals are believed to be the reservoirs of the ESBL genes, as bacteria harbouring these genes have been isolated with increasing frequency from a variety of food animals and their products.[1] Therefore, the potential zoonosis of ESBL-encoding bacteria from food animals has deservedly garnered considerable attention. However, few studies have focused on the characterization of ESBL-producing *E. coli* (ESBL-EC) from dairy cows on a regional scale in China.[2,3] The aim of this study was to investigate the prevalence and molecular characteristics of ESBL-EC in faecal samples collected from dairy cows distributed among the 10 provinces of China. Isolates that carried *mcr-1* were also examined to shed light on the mechanisms through which colistin resistance is achieved.

Materials and methods

Bacterial isolation and identification

In the period between April and June 2016, 10 randomly selected dairy cow farms were enrolled, each in a different city, based on their geographical distribution throughout China (Figure S1, available as Supplementary data at *JAC* Online) and the size of the farms (at least 200 cows per breeding site). The cows enrolled in this study were all between 1 and 3 years old. A total of 651 fresh faecal samples were randomly collected from healthy individuals (Table S1). All faecal samples were kept cool during transportation to the laboratory and were processed within 72 h after sampling. Isolates of ESBL-EC were enriched from the faecal samples using a previously published procedure as described by Schmid *et al.*[4] The enriched solutions were subsequently cultured on chromID ESBL agar plates (bioMérieux, Marcy-l'Étoile, France) for 18–24 h at 37°C to identify presumptive ESBL-producing isolates. The resulting strains were then isolated and characterized by MALDI-TOF MS (Bruker, Bremen, Germany).

Further confirmation of ESBL producers was performed using a double-disc diffusion test, according to the protocol outlined by the CLSI.[5]

Antimicrobial susceptibility testing

The VITEK®2 system, in conjunction with an AST-GN16 card (bioMérieux) was used to perform antimicrobial susceptibility testing. The screening panel consisted of 18 antibiotics (amikacin, amoxicillin/clavulanic acid, ampicillin, aztreonam, cefazolin, cefepime, cefoxitin, ceftriaxone, ciprofloxacin, ertapenem, gentamicin, imipenem, levofloxacin, nitrofurantoin, piperacillin/tazobactam, tigecycline, tobramycin and trimethoprim/sulfamethoxazole). The MICs of colistin for the MCR-1-positive isolates, as well as their transconjugants, were determined using the broth microdilution method. The results were interpreted according to the CLSI criteria.[5] The *E. coli* standard reference strain, ATCC 25922, was used as a quality control.

Detection of resistance genes and MLST

Sequencing of PCR products was used to characterize the ESBL-associated genes, in addition to the *mcr-1* gene.[6] Specific $bla_{\text{CTX-M}}$ alleles were identified using Basic Local Alignment Search Tool (BLAST) analysis. The clonal relatedness of the ESBL-EC isolates was assessed using previously published protocols (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). Genotypic relationships among the isolates were established through the generation of minimum spanning trees using BioNumerics 7.6.

WGS

WGS was performed on 3 *mcr*-positive and an additional 14 randomly selected isolates based on geographical location and on MLST results using the Illumina HiSeq platform (Illumina, San Diego, CA, USA). Sequencing data were assembled using the CLC Genomics Workbench v. 8.0 (QIAGEN, Hilden, Germany). Analyses of the resistome and plasmid replicons were carried out using the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk). The detection of SNPs was performed as previously described.[7] Phylogenetic relationship analysis among these strains was conducted using a total of 99 114 SNPs extracted from 3003 core gene sequences. The phylogenetic tree was constructed using RAxML with 100 bootstrap replicates in the GTRCAT model.[8]

Plasmid characterization

Southern blot and S1-PFGE analyses were performed to estimate the size of the *mcr-1*-carrying plasmids.[9] The transferability of the *mcr*-carrying plasmids was assessed by conjugation.[6] The sequence of the plasmid carrying the *mcr-1* gene, from isolate JL-F4-1 (named pMCR-JLF4), was assembled from the WGS data using plasmidSPAdes.[10] The remaining sequence gaps were filled in by performing combinatorial PCR to amplify the missing regions, followed by Sanger sequencing of the amplicons. The contigs could then be fully assembled into a complete plasmid sequence, which was annotated using the RAST tool.[11]

The sequence of pMCR-JLF4 was compared against other NCBI-accessioned plasmids using BLAST and then plotted by the BLAST Ring Image Generator (BRIG).[12]

GenBank accession numbers

The Whole Genome Shotgun BioProject for the ESBL-EC isolates has been deposited at DDBJ/EMBL/GenBank under BioProject accession no. PRJNA433057. The complete sequence of pMCR-JLF4 has been deposited at DDBJ/EMBL/GenBank under accession no. MH176237.

Results and discussion

In total, 290 isolates from 284 samples were observed to be ESBL-EC as determined by phenotypic methods, giving a carriage rate of 43.6% (284/651). In six of the animals, two distinct ESBL-producing isolates were identified. Among these ESBL-EC isolates, total resistance to ampicillin was observed (100%), followed by very high incidences of resistance to cefazolin (96.2%) and ceftriaxone (96.2%) (Table S2). All isolates were susceptible to piperacillin/tazobactam, imipenem, amikacin and tigecycline. Only one isolate was observed to be resistant to ertapenem. Among the 290 ESBL-EC isolates characterized in the present study, the *mcr-1* gene was detected in 1/24 (4.2%) from Changchun and 2/62 (3.2%) from Xingwen (Table S1).

Of the ESBL-EC isolates, CTX-M alleles were observed in 94.1% (273/290) of all isolates, with the CTX-M group 9 being the most frequently detected (Table S3). The most prevalent genotypes observed in this study were $bla_{\text{CTX-M-14}}$ ($n = 70$; 24.1%), followed by $bla_{\text{CTX-M-15}}$ ($n = 62$; 21.4%), $bla_{\text{CTX-M-17}}$ ($n = 56$; 19.3%), $bla_{\text{CTX-M-55}}$ ($n = 41$; 14.1%) and $bla_{\text{CTX-M-98}}$ ($n = 25$; 8.6%). In addition, eight ESBL-EC carried two CTX-M alleles.

The MLST results revealed extensive diversity among ESBL-EC, with a total of 79 different STs observed. Among the 79 STs, 22 were novel STs and identified for the first time in this study. The minimum spanning tree constructed from the sequencing data did not reveal obvious relationships between the observed STs (Figure 1). The most prevalent STs were ST1141 (10.3%, 30/290), ST10 (8.6%, 25/290), ST107 (6.9%, 20/290) and ST873 (6.9%, 20/290). A member of clonal complex (CC) 10, *E. coli* ST1141 was the major complex observed here. The presence of CC10 *E. coli* has been commonly reported in various samples including animal faeces, waste water and river water from throughout China.[13] Interestingly, all isolates of ST1141 were collected in Datong, all ST50 isolates were collected in Yining and 95.45% (21/22) of the ST515 isolates were collected in Wuzhong. These data suggest clonal spread of ESBL-EC in the individual farms (Figure 1a). The major carrier of the CTX-M-14 allele was ST155 ($n = 13$) and those of the CTX-M-15 and CTX-M-17 alleles were ST515 ($n = 20$) and ST1141 ($n = 30$), respectively. Of particular concern is that the CTX-M-14 alleles were carried by 29 different STs collected in seven provinces. This suggests that *E. coli* strains carrying the CTX-M-14 allele spread in different clonal populations and are now widespread in China.

All of the isolates sequenced in the present study harboured a variety of antimicrobial resistance genes (Table S4). Of note, 11 isolates were observed to be carrying the florfenicol resistance gene, *floR*. Since florfenicol is widely used in dairy cows, this finding underscores the potential adverse effects that the dissemination of the *floR* gene can have on the dairy industry. Interestingly, nine isolates carried the serum survival gene *iss*, which has been previously shown to be an essential virulence factor for enteropathogenic *E. coli* (EPEC).[14] In addition, four isolates carried the EAST-1 heat-stable toxin gene *astA*, a gene associated with enteroaggregative *E. coli* (EAEC). Phylogenetic analysis grouped the 17 sequenced ESBL-EC into two major clusters. The first cluster comprised 12 STs and the second comprised a single ST1704 isolate and two novel STs (Figure S2).

Southern blot and S1-PFGE analyses demonstrated that the *mcr-1* gene from isolate JL-F4-1 was present on a plasmid >55 kb

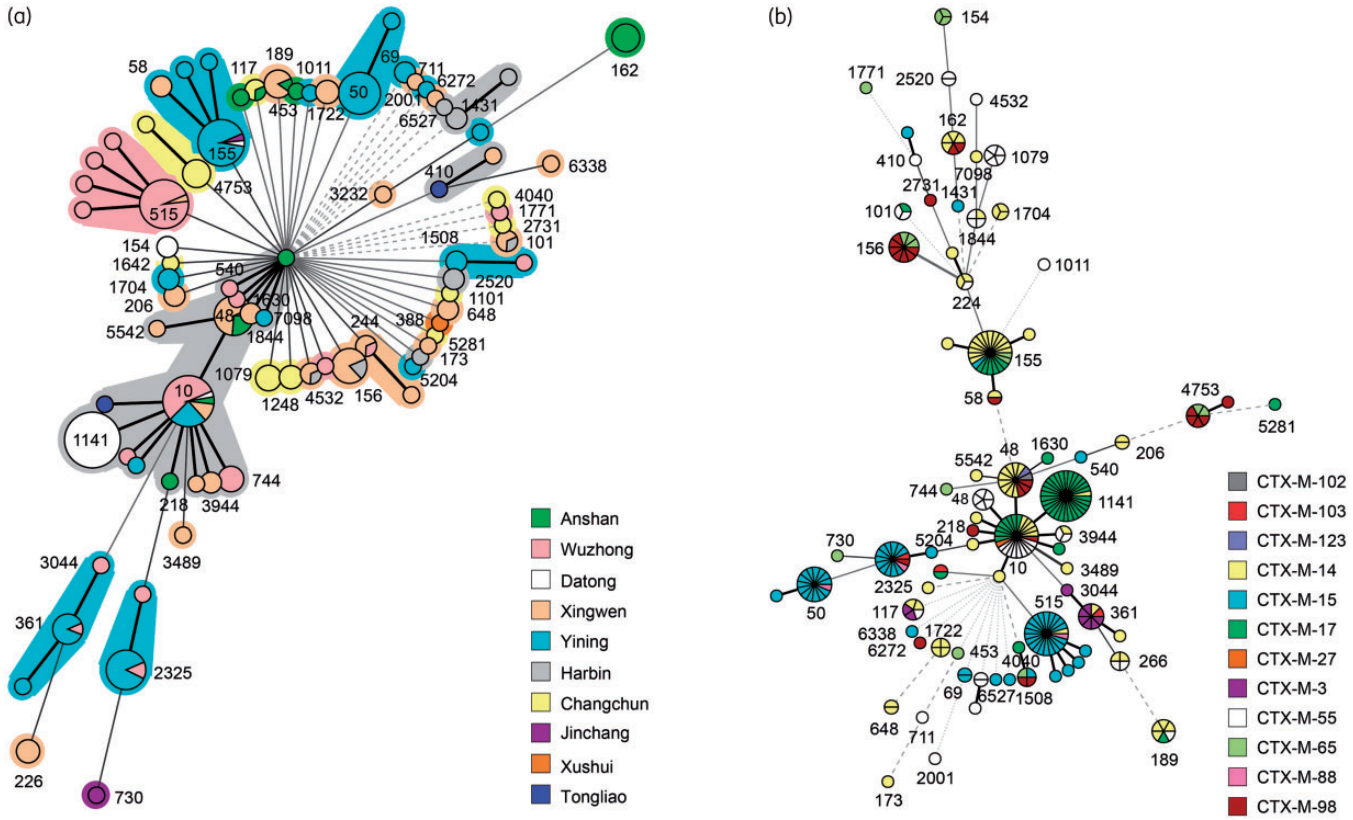

Figure 1. Minimum spanning trees of ESBL-producing *E. coli* isolates based on MLST genotypes. (a) The tree is based on the degree of allele sharing as determined by MLST analysis of 290 ESBL-EC isolates. Shadow zones indicate that >1 ST belongs to the same CC. (b) The tree is based on the *E. coli* isolates containing bla_{CTX-M}. The numbers indicate the most prevalent STs. Each circle represents a genotype and the partitions within a circle represent individual isolates. Black connecting lines represent single-locus variants, whereas dashed lines correspond to double-locus variants. Dotted lines represent 3–7 allele differences between the STs. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

in size (Figure S3). However, Southern blot of SC-F24-1 and SC-F25-1 produced negative results. plasmidSPAdes analysis of WGS data further revealed that *mcr-1* was located on a contiguous sequence ~66 kb in length from isolate JL-F4-1 and was also present on a contig of ~107 kb in isolates SC-F24-1 and SC-F25-1. In addition, PlasmidFinder indicated that the *mcr-1* gene was carried in the IncI2 plasmid in JL-F4-1. Conversely, no plasmid replicon sequences were observed in *mcr-1*-containing contigs from SC-F24-1 and SC-F25-1. These data suggest the possibility that the *mcr-1* gene may be chromosomally encoded in the SC-F24-1 and SC-F25-1 isolates.

The complete sequence of the 66.7 kb plasmid pMCR-JLF4 was BLASTed against the nr/nt database. An overall nucleotide identity (99%) with query coverages of 97%–100% to pJIE3685-1 (KY795978), pBA77-MCR-1 (KX013539) and pCREC-527 (KY657476) were observed (Figure 2). Among these plasmids, the *mcr-1* gene was annotated within the same genetic context, with the order *nikA–nikB–mcr-1–pap2*. Interestingly, this structure was also identified in a clinical isolate of *E. coli* from a septicaemic patient in a study previously published by our group.[15] By contrast, most of the recently reported *mcr-1*-containing plasmids have been observed to be closely associated with IS*Apl1*.[16] Conjugation analysis confirmed that the *mcr-1* gene in pMCR-JLF4 was transferable without the assistance of the IS*Apl1* gene. Further analyses revealed four coding sequence insertions in pMCR-JLF4 (Figure 2 and Figure S4). The 59140–64816 bp insertion region was observed to carry genes encoding three hypothetical proteins as well as the transposable element Tn*Ec1* (Figure 2). At the time of manuscript preparation, a limited number of studies had reported on the low prevalence of MCR-1 in cattle or adult cows.[17,18] These observations published elsewhere are consistent with the observations reported here. To the best of our knowledge, this is also the first report of the complete nucleotide sequence of plasmids carrying the *mcr-1* gene from cows in China.

In conclusion, the data presented here suggest a high prevalence of ESBL-EC in cow farms in China. Furthermore, a high level of ESBL-EC isolates was observed, with CTX-M-14 and CTX-M-15 being the predominant alleles. The association of ESBL-EC with colistin resistance is of particular concern, although a low prevalence of MCR-1-producing ESBL-EC was detected. This study was conducted exclusively on large-scale cow farms in selected regions. Therefore, these data may not be truly representative of the entirety of China and further studies are required to increase confidence in the findings.

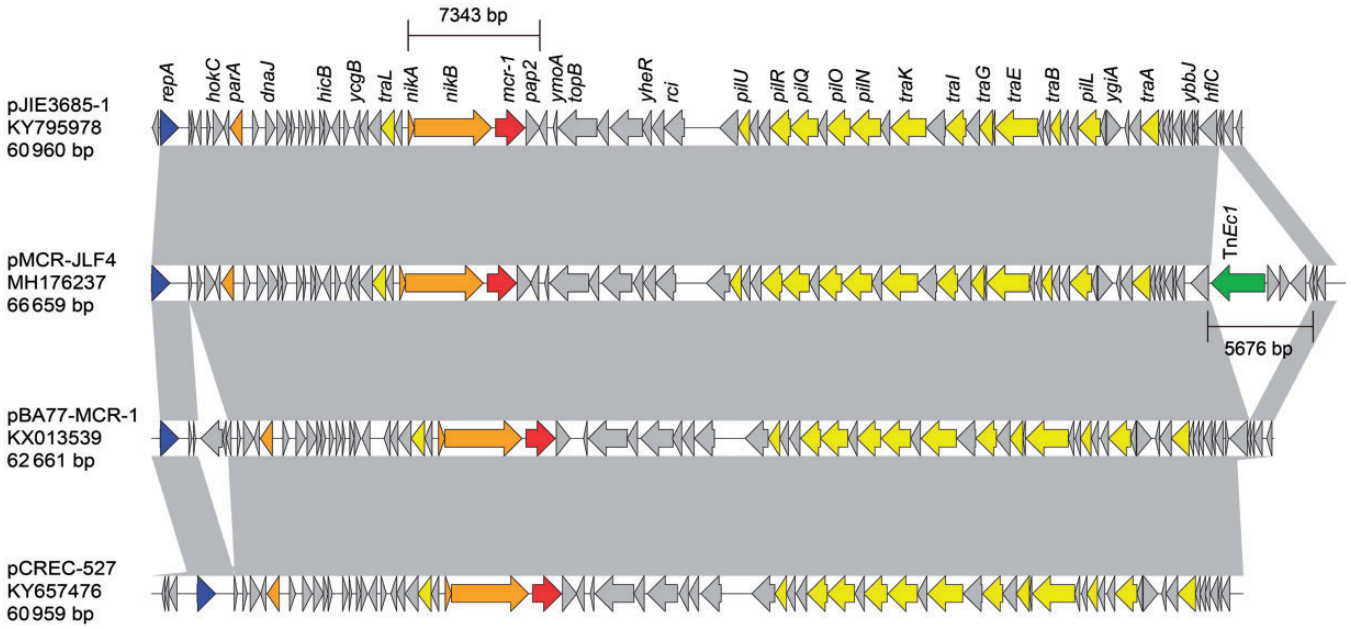

Figure 2. Linear plasmid characterization and comparison of MCR-1-producing IncI2 plasmids pMCR-JLF4 (MH176237, this study), pJIE3685-1 (KY795978), pBA77-MCR-1 (KX013539) and pCREC-527 (KY657476). Grey shading indicates shared regions with a high degree of nucleotide identity (70%–100%). Arrows represent coding sequences and the associated direction of transcription. Arrow sizes are proportional to gene length. Antimicrobial resistance genes are shaded red. Mobile elements are represented by green. Blue arrows indicate genes associated with replication. Genes associated with plasmid stability are orange. Yellow arrows indicate genes involved in conjugation. Grey arrows represent genes encoding hypothetical proteins and proteins of unknown function. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

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Transparency declarations

None to declare.

Supplementary data

Tables S1–S4 and Figures S1–S4 are available as Supplementary data at *JAC* Online.

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