First Isolation and Molecular Characterization of \textit{bla} CTX-M-121 -producing \textit{Escherichia coli} O157:H7 Strain Y4-A109 from Cattle in China

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Abstract

Background: To study the antibiotic resistance, the molecular epidemiology of bovine Escherichia coli (E. coli) O157:H7, and exploring the intrinsic relationship among different isolates, we have collected 27 bovine E. coli O157:H7 strains in Xinjiang from 2012 to 2017 and evaluated virulence genes, antibiotic resistance, and pulsed-field gel electrophoresis (PFGE) molecular typing.

Results: Of all the 27 bovine E. coli O157:H7 strains analyzed, 21 strains contained at least one virulence gene, 19 strains carried eae gene (70.4%) and 8 of them carrying stx1 + stx2 + eae + hly + tccP. Most strains were sensitive to all the antibiotics tested. However, 4 of which were antibiotic-resistant, and 2 of which possessed multi-drug resistance, including one ESBL-producing strain. This is the first report of the bla CTX-M-121 gene in bovine E. coli O157:H7. Moreover, the bla CTX-M-121 gene can be transmitted horizontally through plasmid between strains. The similarity of PFGE spectra of 27 strains was between 65.8% and 100%. Two types of PFGE were obtained through cluster analysis, including clusters I and II.

Conclusions: E. coli O157:H7 may have undergone clonal propagation in cattle farms as well as cross-regional transmission and horizontal transmission in different regions in Xinjiang China.

Background

E. coli O157:H7 is a major foodborne pathogen that causes severe bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans [1]. E. coli O157: H7 was first recognized as a pathogen in an investigation of an outbreak of hemorrhagic colitis associated with hamburger consumption in 1982 [2]. Since then, many outbreaks of E. coli O157:H7 infection have been reported in the United States, Canada, Japan, and China [3-
Cattle is reported to be the major reservoir and source of infection for *E. coli* O157:H7. *E. coli* O157:H7 from healthy cattle has been reported worldwide [7]. The infected cattle irregularly excreted *E. coli* O157:H7 without any pathological symptom, and transmitted the pathogen to humans through food, water, direct contact with animals or the environment [8].

The pathogenicity of *E. coli* O157:H7 is associated to genes encoding for multiple virulence factors. Shiga toxins (stx) is one of the major virulence factors involved in the pathogenesis of *E. coli* O157:H7 and is encoded by the stx1 or stx2 genes [9]. Intimin and enterohemolysin (encoded by the eae gene and the hly gene, respectively) are two other markers that play a major role in pathogenesis [9]. The tccP protein encoded by the tccP gene is a pathogenic molecule of *E. coli* O157:H7 and is transduced into host cells through the type III secretion system to exert its pathogenic effect [10]. These genetic virulence characteristics are commonly used in epidemiological studies of strains from various origins [6,11].

Undoubtedly, antimicrobials are the main tool for the prevention and treatment of bacterial diseases in animals. However, antibiotic resistance has become a serious problem worldwide, especially in developing countries where the quality, distribution and use of antibiotics in human medicine and veterinary medicine are not strictly controlled [11, 12]. Diseases caused by *E. coli* usually require antimicrobial treatment, but antibiotic-resistant strains of this bacterium may cause more chronic and more severe diseases than their antibiotic-susceptible counterparts [12]. *E. coli* O157: H7 strains isolated from humans and animals have been resistant to a variety of antibiotics [13]. The emergence of multi-drug resistant (MDR) *E. coli* O157:H7 is a public health issue.

Xinjiang has one of China’s largest cattle raising industry. Effective prevention and control of bovine pathogenic microorganisms is a prerequisite to ensure the healthy and
sustainable development of the cattle industry and consumer safety. To further assess the potential public health impact of *E. coli* O157:H7 isolates, we investigated the pathogenicity and antibiotic resistance of these strains originating from farms and slaughterhouses, and examined the intrinsic relationship among different isolates and assessed the potential dissemination of MDR profiles *in vitro*.

**Results**

**Isolation of *E. coli* O157:H7**

A total of 27 *E. coli* O157:H7 strains were isolated from 2,657 cattle samples in Xinjiang, 2 of which were collected from one carcass swab sample, 4 from 3 feed samples, 8 from 8 feces samples and 13 from 5 rectal swab samples, and multiple colonies were selected from the same plate (Table 1).

**Presence of virulence genes**

Of the 27 *E. coli* O157:H7 isolates tested, 21 isolates carried more than one virulence genes, and 6 (22.2%) did not encode the genes evaluated in the study. Polymerase chain reaction (PCR) showed that 2 (7.4%) isolates carried *stx*1, 8 (29.6%) possessed *stx*2, and 8 (29.6%) contained both *stx*1 and *stx*2. The *eae* gene and *hly* gene were detected in most (70.4%) and 17 (63.0%) *E. coli* O157:H7 strains, respectively. *TccP* in combination with *hly* and *eae* was found in 15 (55.6%) isolates, *stx*2 alone and *eae* alone were present in 2 (7.4%) isolates (Table 1).

**Antibiotic resistance spectrum and distribution of antibiotic resistance genes**

Twenty-three (85.2%) *E. coli* O157:H7 isolates were sensitive to all of antimicrobials investigated. Four isolates (14.8%) were resistant, 3 of which were isolated from the same cattle farm in Yili. Of the four resistant isolates, 2 were only resistant to tetracycline, and one of which carries *tetA* gene that encodes a tetracycline efflux pump. The other two were MDR strains with the resistant patterns: AMP/CHL/CIP/CTX/LEV /PIP/SXT/TET (Y4-C21-
1) and AMP/CAZ/CHL/CIP/CTX/LEV/PIP/SXT/TET (Y4-A109). In particular, the ones with the Y4-A109 was an Extended Spectrum Beta-Lactamases (ESBLs)-producing strain and carrying the $bla_{CTX-M-121}$ gene.

**Transferability of $bla_{CTX-M}$ genes and plasmid replicon typing**

The $bla_{CTX-M}$ gene of *E. coli* O157:H7 isolate (Y4-A109) was transferred to the recipient strain (azide-resistant *E. coli* J53) by conjugation at frequencies of $10^{-6}$ per donor cell. Resistance to ampicillin, cefotaxime, ceftazidime, trimethoprim- sulfamethoxazole and tetracycline, and resistance to the $bla_{CTX-M-121}$ gene from the $bla_{CTX-M}$-producing O157:H7 isolate can be transferred to the recipient. The $bla_{CTX-M-121}$ gene is carried by non-typeable plasmid.

**Epidemiological typing**

The chromosomal DNA of 27 isolates was available for PFGE typing and the isolates showed 14 different PFGE profiles (Fig. 1). The similarity among the types was higher than 65.8%, with the two dominant clusters I and II accounting for 40.7%, and 18.5%, respectively. Cluster I mainly includes type p4, and cluster II mainly consists of type p11 and p12. Nine strains of type p4 and 5 strains of type p12 were highly consistent in sampling time and location, which was determined as clonal propagation. Significant differences were found between p4 and p12 strains ($p$ value?) with isolates were collected in different regions and years. Four drug-resistant bacterial strains belong to four different types.

**Discussion**

*E. coli* O157:H7 is an important foodborne pathogen [1]. Cattle is considered to be the major reservoir and transmitting diseases to humans primarily by eating contaminated food. In this study, a total of 2,657 cattle sourced samples were collected from Tacheng (2
farms), Bole (1 farm), Yili (4 farms and 1 slaughterhouse), Wujiuq (1 farm), Changji (2 farms), Wulumuqi (4 farms) and Akesu (4 farms), 27 \( E. coli \) O157:H7 strains were isolated. \( E. coli \) O157:H7 was isolated from Yili, Wulumuqi and Akesu, while not from Tacheng, Bole, Wujiuq and Changji, which indicated the presence of regional differences in bacterial distribution. We discovered that the number of \( E. coli \) O157:H7 isolates were low in winter and high in summer, which is consistent with the previous finding [14]. In addition, the number of \( E. coli \) O157:H7 isolates were lower in Xinjiang when compared to other provinces in China [6, 15]. Our previous studies have shown that the immunomagnetic separation (IMS) in practice was not statistically significant different compared to conventional method [16]. The low isolation rate of \( E. coli \) O157: H7 may be related to the severe dry weather conditions of Xinjiang, which needs further confirmation.

The pathogenicity of \( E. coli \) O157:H7 is associated with several virulence factors, including the production of Shiga toxins (\( stx1 \) and/or \( stx2 \)), intimin (\( eae \)), enterohemolysin (\( hly \)) and tir couple cytoskeleton protein (\( tccP \)). The results showed that 37.0% and 59.3% of \( E. coli \) O157:H7 isolates contain \( stx1 \) and \( stx2 \) genes, respectively. Epidemiological researches have shown that the virulence of \( stx2 \)-producing strains is higher than \( stx1 \) producers [17]. The \( eae \) gene which is necessary for the attaching and effacing activity encodes an intimin protein that is essential for pathogenesis [18]. In our study, this important virulence gene was detected in 70.4% of the \( E. coli \) O157:H7 isolates. We identified the \( tccP \) gene in 55.6% of the \( E. coli \) O157:H7 strains. Noticeably, \( tccP \) gene is highly correlated with both \( eae \) gene and \( hly \) gene, but not with the \( stx \) gene.

Although the sample size in the slaughterhouse is small, the isolation rate of carcass swab samples was higher than others samples from the cattle farms, and one of which was the MDR bacteria, which showed co-selection evidence of antibiotic resistance and virulence.

In this study, two \( E. coli \) O157:H7 isolates were found to be resistant against new and
more clinically important antimicrobial compounds such as fluoroquinolones and cephalosporins. Beta-lactamases production is the main mechanism underlying the cephalosporin resistance in Gram-negative bacteria [19]. Broad-spectrum cephalosporins are important drugs in both human and veterinary medicine. We investigated various narrow-spectrum (bla TEM and bla SHV) and extended-spectrum (bla CTX-M) β-lactamase-encoding genes, but only identified one- bla CTX-M. This is the first report of the bla CTX-M-121 gene in bovine E. coli O157:H7. The tetA is one of the most widespread tet genes found in Enterobacteria [20], and is the only tetracycline resistant gene identified in four tetracycline-resistant strains. However, to our knowledge, this is the first report about the presence of tetA in bovine E. coli O157:H7 in Xinjiang. Conjugative transfer of non-typeable plasmid was observed. Conjugation experiments successfully transduced MDR to β-lactamases, sulfonamides and tetracycline. This study highlights the importance of encouraging the appropriate use of antibiotics.

The dendrogram analysis of the PFGE results showed that the two E. coli O157:H7 strains isolated from the same carcass swab samples from the slaughterhouse belonged to clusters I and II, suggesting that cross-contamination may occur during the slaughter process. The Y4-A20-1, Y4-A20-3, Y4-A20-4 of cluster I, and Y4-A20-5 of cluster II from the same rectal swab, indicates that different E. coli O157:H7 strains have been colonized in cattle. Cluster I W1-E51-5 and cluster II W1-E51-3F were isolated from the same feed sample, suggesting that the cattle farm feed was contaminated with different E. coli O157:H7 strains. Cluster II Y1-166 and Y3-F328 were isolated from different cattle farms in the same region at the same time, which further proved the horizontal transmission was an important means of E. coli O157:H7 dissemination in these farms. Cluster I Y4-A20-1, W2-A61-2 and W1-E51-5, and cluster II Y2-F25, A1-F13 and A2-F14 were isolated at
different time points and from different regions. These cattle farms were separated far away. Cross-regional transmission of bacteria may be caused by trading in live animals. Based on the analysis of virulence genes and drug resistance of \textit{E. coli} O157:H7, we speculate that virulence and drug resistance may be acquired or lost during the evolution and transfer of the same cluster of strains.

Conclusions

In this study, \textit{E. coli} O157:H7 contamination was found in cattle farms and slaughterhouse in Xinjiang, and most isolates carried at least one virulence gene. \textit{E. coli} O157:H7 may have undergone clonal propagation in cattle farms and transmitted horizontally in different regions.

Methods

\textbf{Sample collection}

Samples (n= 2657) were collected from 18 farms and one cattle slaughterhouse in Tacheng, Bole, Yili, Wujiaqu, Changji, Wulumuqi and Akesu in Xinjiang of China between October 2012 and March 2017, including 1155 fresh feces, 1236 rectal swabs, 110 feed, 108 water and 48 carcass swabs (Table 2).

\textbf{Bacterial isolate}

Each 1 g or 1 ml sample (feces/feed/water) were aseptically added to 9 ml of trypticase soya broth (TSB) containing 20 mg/l novobiocin and were incubated for 6-8 h at 37 °C. A rectal swab was transferred into a separate tube containing 2 ml nutrient broth and cultured at 37 °C for 24 h [21]. One carcass swab was put into a stomacher bag and added 500 ml of modified trypticase soya broth containing 8 mg/l novobiocin. Each sponge was mixed in the stomacher bag for 2 min and then incubated for 20 h at 37 °C [22]. This was streaked out onto Sorbitol MacConkey agar supplemented with 0.01mg/l cefixime and
0.5mg/l potassium tellurite (Haibo, Qingdao, China) (CT-SMAC) and incubated for one day at 37 °C. One or more pale colonies were individually selected as presumptive E. coli O157 per sample. The prevalence of E. coli O157:H7 was assessed via polymerase chain reaction (PCR) (rfbE and fliC genes [23]) (Table 3). The positive isolates were each inoculated into separate TSB and incubated for one day at 37 °C, from which glycerol stock was made and then stored at -80 °C for further analysis.

**Virulence analysis of isolates**

**DNA extraction**

DNA was extracted by boiling the isolates. Each colony was inoculated on CT-SMAC and incubated for 16 h at 37 °C to obtain fresh colony. Several colonies were selected and suspended separately in 200 μl of sterile distilled water in 1.5ml eppendorf tubes. The suspensions were then boiled at 95 °C for 10 min in a water bath. After centrifuging at 12000 rpm for 10 min, the supernatant containing the template DNA was transferred into 1.5ml Eppendorf tubes without nuclease and were stored at -20 °C until use.

**Determination of virulence genes by PCR**

To characterize the virulence genes, amplification products of stx1, stx2, eae, hly and tccP genes were used, which encode for Stx1, Stx2 toxins, intimin, enterohemolysin, and tir couple cytoskeleton protein respectively. The primers, conditions and references cited are listed in Table 3. Amplification of the targeted gene used EX Taq (Takara, Dalian, China) with the following PCR program: 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Adjust annealing temperature according to primer Tm value (Table 3). The PCR amplicons (10 μl) were subjected to electrophoresis on a 1.2% agarose gel in 1× TAE buffer at 115 V for 30 min, and stained with SYBR Green (Fermentas, Germany).

**Antimicrobial susceptibility tests**
The susceptibility of to antibiotics was tested using the Kirby-Bauer disc diffusion technique. Antibiotic discs obtained from OXOID, UK, including ampicillin (AMP), piperacillin (PIP), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), ampicillin-sulbactam (SAM), piperacillin-tazobactam (TZP), amoxicillin-clavulanic acid (AMC), gentamicin (GEN), amikacin (AMI), streptomycin (STR), trimethoprim-sulfamethylisoxazole (SXT), chloramphenicol (CHL), levofloxacin (LEV), ciprofloxacin (CIP), tetracycline (TET), and polymyxin B (PB) [26]. *E. coli* ATCC25922 was used as a quality control strain in the susceptibility tests. The ESBLs-producing isolates were determined by double-disk synergy tests according to CLSI [26].

**Detection of antibiotic resistance genes**

The following resistance determinants were investigated by PCR: *bla*<sub>CTX-M</sub> (the CTX-M-type genes were detected using universal primers *bla*<sub>CTX-M-U</sub> [27], and the entire CTX-M-type genes were amplified using the primers *bla*<sub>CTX-M-1G</sub> [27], *bla*<sub>CTX-M-2G</sub> [28] or *bla*<sub>CTX-M-9G</sub> [29]), *bla*<sub>TEM</sub> [30], and *bla*<sub>SHV</sub> [30] which encode β-lactamases; chloramphenicol (*cmlA1* [31]) efflux pumps; sulfonamide resistance gene (*sul1* [32]); and the *tetA* [33], *tetE* [33], and *tetG* [33] tetracycline efflux pumps. Primer for the different genes are listed in (Table 3). Purified PCR products were sequenced. The DNA sequences and deduced amino acid sequences were compared with sequences reported in GenBank to confirm the subtypes of the β-lactamase gene.

**Conjugation experiments and plasmid analysis**

Sodium azide-resistant *E. coli J53* was used as a recipient and conjugated to a *bla*<sub>CTX-M</sub>-producing isolate by filtration. Transconjugants were selected on Mac Conkey agar containing cefotaxime or ceftazidime (4 μg/ml) and sodium azide (200 μg/ml). ESBLs and antibiotic susceptibility were also tested in selected transconjugants, and the presence of
bla genes was determined using PCR as described above. The resistance plasmids carried by transconjugants were typed by using PCR-based replicon typing [34].

**Epidemiological typing**

All available isolates were characterized by pulsed field gel electrophoresis (PFGE) using the CHEF-MAP-PER System (Bio-Rad Laboratories, Hercules, CA, USA) as described by Gautom [35]. Briefly, chromosomal DNA of *E. coli* O157:H7 isolate was isolated and the inserts were digested with *XbaI* (TaKaRa Dalian, China) for 16 h at 37 °C. The electrophoresis was performed at 6.0 V/cm for 18.5 h with an angle of 120° at 14°C. The pulse time was increased from 0.5 to 60 s. The Salmonella serotype Braenderup H9812 (ATCC BAA-664) was chosen as the molecular weight marker. Gels were then stained in ethidium bromide (1.0 mg/L). The results were interpreted according to the criteria of Tenover et al. [36].

**Abbreviations**

AMC: Amoxicillin-clavulanic acid
AMI: Amikacin
AMP: Ampicillin
ATM: Aztreonam,
CAZ: Ceftazidime
CHL: Chloramphenicol
CIP: Ciprofloxacin
CT-SMAC: Sorbitol MacConkey agar containing cefixime and potassium tellurite
CTX: Cefotaxime
*E.coli: Escherichia coli*
ESBLs: Extended Spectrum Beta-Lactamases
FEP: Cefepime
GEN: Gentamicin
IMS: immunomagnetic separation
LEV: Levofloxacin
MDR: Multi-drug resistant
PCR: Polymerase chain reaction
PFGE: Pulsed-field gel electrophoresis
PB: Polymyxin B
PIP: Piperacillin
SAM: Ampicillin-sulbactam
STR: Streptomycin
stx: Shiga toxins
SXT: Trimethoprim-sulfamethylisoxazole
TET: Tetracycline
TSB: Trypticase soya broth
TZP: Piperacillin-tazobactam

Declarations

Ethics approval and consent to participate
The study was carried out on private land, no specific permissions were required for these locations. Sampling and publication of the data were approved by the farm owners. All procedures performed on the cattle were approved by the Animal Care and Use Committee of Xinjiang Agricultural University.

Consent for publication
Not applicable.

Availability of data and materials
Not applicable.
Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Z.Q.S. and P.P.T. conceived and designed the experiments. L.Z., M.M.Z., D.W., and K.Q.M. performed the experiments. Y.Z. and Y.Y.L. analyzed the data. P.P.T., L.N.X., and J.X.X. contributed to the writing of the manuscript. All authors read and approved the article.

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Tables

| Sample place | Farm | Sample source | Strain     | Virulence genes            |
|--------------|------|---------------|------------|----------------------------|
| Yili         | A    | Feces         | Y2-F25     | stx2                       |
|              |      |               | Y2-F27     | stx2                       |
|              | B    | Feces         | Y1-F166    | stx1+stx2+eae+hly+tccP     |
|              | C    | Feces         | Y3-F328    | stx2+eae+hly+tccP          |
|              | D    | Rectal swab   | Y4-A20-1   | stx1+stx2+eae+hly+tccP     |
|              |      |               | Y4-A20-2   | stx1+stx2+eae+hly+tccP     |
|              |      |               | Y4-A20-3   | eae                        |
|              |      |               | Y4-A20-4   | stx1+stx2+eae+hly+tccP     |
|              |      |               | Y4-A20-5   | eae                        |
|              |      |               | Y4-A41-2   | stx1+stx2+eae+hly+tccP     |
|              |      |               | Y4-A41-4   | stx1+eae+hly               |
|              |      |               | Y4-A103    | eae+hly                    |
|              |      |               | Y4-A109    | stx1+stx2+eae+hly+tccP     |
| Slaughterhouse | Carcass swab | Y4-C21-1    | stx1+stx2+eae+hly+tccP     |
|              |      |               | Y4-C21-2   | stx1+stx2+eae+hly+tccP     |
Table 2 Information on sample collection

| Source of sample | NO. of farms | Feces | Rectal swab | Water | Feed | Carcass swab | NO. of positive samples |
|------------------|--------------|-------|-------------|-------|------|--------------|------------------------|
| Tacheng          | 2            | 134   | 0           | 0     | 0    | 0            | 0                      |
| Bole             | 1            | 43    | 82          | 10    | 6    | 0            | 0                      |
| Yili             | 4            | 480   | 397         | 42    | 29   | 0            | 8                      |
| Wujiq            | 1 slaughterhouse | 0 | 0 | 0 | 48 | 0 | 1 |
| Changji          | 2            | 46    | 211         | 17    | 23   | 0            | 0                      |
| Wulumuqi         | 4            | 90    | 467         | 30    | 48   | 0            | 4                      |
| Akesu            | 4            | 354   | 0           | 0     | 0    | 0            | 4                      |
| Total            |              | 1155  | 1236        | 108   | 110  | 48           | 17                     |

—, No virulence genes were identified in this study.

Table 3 Oligonucleotides of the various targeted genes
| Target gene | Primer Sequence (5'-3') (Forward/reverse) |
|-------------|----------------------------------------|
| rfbE        | ATTGCCGTGAAGCCTTTG/CGAGTACATTGCCATCGTG |
| fliC        | GCCGTCTGAGGTCTATCGAGC/CAACGCTGACTTTATGCCATCC |
| stx1        | GAAGAGTCCGGTGGATTACG/AGCGATGCAGCTTTAAATA |
| stx2        | TTAACCCACCCACGCGCAGT/GCTCTGGATGCACTCTGT |
| eae         | CATTATGGAACGGCAGAGGT/ACGGATATCGAAGCCATT |
| hly         | CAACCGGAGCTTATATTTCTGTCA/AATGTTATCCCATGACATCATTTGACT |
| tccP        | CGCCATATGATTAAATGTTTCCTCAC/CTCGAGTACGAGCGCTTAGATGATT |
| blaCTX-M-U  | ATGTGCAAYACCAGTAARGT/TGGGTRAARTARGTSACCAG |
| blaCTX-M-1G | GTTACAATGTGAGAAGCAG/CCGTTTCGCCATTAACAA |
| blaCTX-M-2G | ATGATGACTCAGAGCATTCCG/TGGGTTACGATTTTCGCC |
| blaCTX-M-9G | ATGTTGACAAGAGAGTGCA/CCCTCGGAGATTTCTC |
| blaTEM      | ATGAGTATTCAACACATTTCGG/TTACCAATGCTTAATCAG |
| blaSHV      | CCGGGTTATTCCTATTTCTGCT/TAGCGTTGCCAGTGTC |
| cmlA1       | CCGCCAGGGTGTGTTGTGTATC/CACCGGCTCGCCATCATT |
| sul1        | CGGGGTTTTCTGCTAGGC/ATCCATGCGTGAGGTTTC |
| tetA        | GCTACATCCTGCTTGCCCT/CATAGATCGCCGCTGAGAG |
| tetE        | AACACACATCCCTCACTACGC/AAATAGGCCACAAAGCTC |
| tetG        | GTGCGGTACGATCTCTGCAG/AGCAACAGAAATCGGGAACAC |

Figures
Fig. 1 Dendrogram of Xbal pulsed-field gel electrophoresis profiles of O157:H7 isolates.

Dendrogram of Xbal pulsed-field gel electrophoresis profiles of E.coli O157:H7 isolates