He et al. *Zool. Res.* 2021, 42(4): 461−468
https://doi.org/10.24272/j.issn.2095-8137.2021.153

ZR | Zoological Research

Article Open Access

Clonal spread of *Escherichia coli* O101:H9-ST10 and O101:H9-ST167 strains carrying *fosA3* and $bla_{CTX\text{-}M\text{-}14}$ among diarrheal calves in a Chinese farm, with Australian *Chroicocephalus* as the possible origin of *E. coli* O101:H9-ST10

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ABSTRACT

During a 2018 antimicrobial resistance surveillance of *Escherichia coli* isolates from diarrheal calves in Xinjiang Province, China, an unexpectedly high prevalence (48.5%) of fosfomycin resistance was observed. This study aimed to reveal the determinants of fosfomycin resistance and the underlying transmission mechanism. Polymerase chain reaction (PCR) screening showed that all fosfomycin-resistant *E. coli* carried the *fosA3* gene. Pulsed-field gel electrophoresis (PFGE) and southern blot hybridization revealed that the 16 *fosA3*-positive isolates belonged to four different PFGE patterns (i.e., A, B, C, D). The *fosA3* genes of 11 clonally related strains (pattern D) were located on the chromosome, while others were carried by plasmids. Whole-genome and long-read sequencing indicated that the pattern D strains were *E. coli* O101:H9-ST10, and the pattern C, B, and A strains were O101:H9-ST167, O8:H30-ST1431, and O101:H9 with unknown ST, respectively. Among the pattern C strains, the $bla_{CTX\text{-}M\text{-}14}$ gene was co-localized with the *fosA3* gene on the F18:A-:B1 plasmids. Interestingly, phylogenetic analysis based on core genome single nucleotide polymorphisms (cgSNPs) showed that the O101:H9-ST10 strains were closely related to a Australian-isolated *Chroicocephalus*-origin *E. coli* O101:H9-ST10 strain producing CTX-M-14 and FosA3, with a difference of

Received: 30 April 2021; Accepted: 15 June 2021; Online: 22 June 2021

Foundation items: This work was supported by the National Natural Science Foundation of China (31625026), International Science and Technology Cooperation Project of Xinjiang Production and Construction Corps (XPCC) (2019BC004), and Innovation Team Project of Guangdong University (2019KCXTD001)

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only 11 SNPs. These results indicate possible international dissemination of the high-risk *E. coli* clone O101:H9-ST10 by migratory birds.

Keywords: Clonal spread; Bovine; *fosA3*; $bla_{CTX\text{-}M\text{-}14}$; O101:H9-ST10; *Chroicocephalus*

INTRODUCTION

Bacterial infections in domesticated bovines continue to increase year by year (Ruegg, 2017). Diarrhea in calves, which is partly caused by pathogenic *Escherichia coli*, is one of the three major bovine diseases causing economic loss to cattle producers (Wieler et al., 2007). Antimicrobials are often used to treat calf diarrhea caused by pathogenic *E. coli* (Constable, 2004). However, due to the abuse and misuse of antimicrobials, antimicrobial resistance (AMR) among bovine *E. coli* has become an important issue, especially for bacterial disease treatment and public health. Interestingly, in many cases, antibiotic resistance in cattle-origin *E. coli* is lower than in that originating from pigs or chickens (Ho et al., 2011; Li et al., 2019).

As an old antibiotic used in the treatment of uncomplicated urinary tract infections, fosfomycin has been reintroduced with other antimicrobials for the clinical treatment of multidrug-resistant (MDR) bacteria due to its excellent antimicrobial activity (Bassetti et al., 2019; Falagas et al., 2016). Although fosfomycin is not approved for animal use in China, fosfomycin resistance is widely reported among food animals nationwide. In addition, the plasmid-mediated *fosA3* gene is reported to be a major determinant of fosfomycin resistance and is often co-localized with CTX-M β-lactamase genes (He et al., 2013, 2017; Huang et al., 2020). Consequently, the *fosA3* gene can be co-selected under the use of β-lactam antibiotics. During AMR surveillance of *E. coli* from a cattle farm in Xinjiang Province, China, an unexpectedly high prevalence (48.5%) of fosfomycin resistance was observed, which was significantly higher than previously reported rates in bovines (Chan et al., 2014; Wang et al., 2017b). Hence, this study aimed to uncover the determinants of fosfomycin resistance and the underlying transmission mechanism in diarrheal calf-derived *E. coli* isolates.

MATERIALS AND METHODS

Bacterial strain

A total of 51 fecal samples were collected from diarrheal calves aged less than one month from a farm located in Yili, Xinjiang, China, in May 2018. These calves had been treated with enrofloxacin, ceftiofur, gentamycin, ampicillin, penicillin, florfenicol, colistin, and tulathromycin. The collected samples were enriched in Luria-Bertani (LB) broth at 37 ℃ for 16–18 h. The overnight culture was then incubated on a MacConkey agar plate. One isolate showing *E. coli* morphology from each sample was further identified using matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF MS) (Shimadzu-Biotech Corp., Japan).

Antimicrobial susceptibility testing and detection of resistance genes

According to the Clinical Laboratory Standard Institute (CLSI) guidelines (M07-A11), the minimum inhibitory concentrations (MICs) of all *E. coli* isolates against fosfomycin with 25 mg/L glucose-6-phosphate, beta-lactams (ampicillin, cefoxitin, ceftazidime, cefquinome, cefotaxime, and imipenem), aminoglycosides (amikacin, streptomycin, apramycin, gentamicin, and neomycin), tetracyclines (tetracycline and doxycycline), florfenicol, trimethoprim-sulfamethoxazole, and ciprofloxacin were determined using agar dilution or broth microdilution methods (colistin and tigecycline). The *E. coli* ATCC 25922 strain was used for quality control. The MICs were interpreted according to the criteria of the CLSI (M100-S30) (for fosfomycin, ampicillin, cefoxitin, ceftazidime, cefotaxime, imipenem, gentamicin, amikacin, tetracycline, doxycycline, trimethoprim-sulfamethoxazole, and ciprofloxacin), EUCAST (http://www.eucast.org) (for colistin and tigecycline), US Food and Drug Administration (FDA) for streptomycin (S, ≤32 mg/L; R, ≥64 mg/L), US National Antimicrobial Resistance Monitoring System (NARMS) for apramycin (S, ≤8 mg/L; R, ≥64 mg/L), and veterinary CLSI (VET06-S1) (for cefquinome, neomycin, and florfenicol).

Polymerase chain reaction (PCR) amplification was used to screen the fosfomycin resistance gene *fosA3* and other important antimicrobial resistance genes (ARGs), including the extended-spectrum beta-lactamase gene $bla_{CTX\text{-}M\text{-}1G/9G}$, AmpC beta-lactamase gene $bla_{CMY\text{-}2}$, 16S rRNA methyltransferase genes *armA* and *rmtB*, florfenicol resistance gene *floR*, and colistin resistance gene *mcr-1*, using previously described primers (Supplementary Table S1) (Cao et al., 2020; Chen et al., 2007; Yan et al., 2004). PCR mapping was used to determine the genetic background of *fosA3* with known primers (Supplementary Table S1) (Hou et al., 2012). The PCR products were subjected to Sanger sequencing (TsingKe Biological Technology, Beijing, China), and the obtained sequences were ascertained without mutation by NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed-field gel electrophoresis (PFGE), S1-PFGE, and southern blot hybridization

The clonal relationship of *fosA3*-positive *E. coli* isolates was assessed based on a rapid PFGE protocol (Gautom, 1997). Total DNA was digested by the *Xba*I enzyme (TaKaRa, Japan), embedded in low-melting-point agarose (Bio-Rad, USA), and subjected to PFGE using the CHEF-MAPPER System (Bio-Rad, USA). The electrophoretic conditions were: initial switch time, 2.16 s; final switch time, 63.8 s; running time, 19 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14 °C; ramping factor, linear. The *Salmonella enterica* serotype Braenderup H9812 was used as a molecular size marker. The gel was dyed with ethidium bromide, visualized using a gel imaging system (Bio-Rad, USA), and analyzed with BioNumerics v6.6 (Applied Maths, Belgium). DNA patterns were interpreted based on proposed criteria (Tenover et al., 1995). The S1-PFGE protocol was the same as that of PFGE, except that total DNA was digested by S1 nuclease (TaKaRa, Japan). The products were subsequently used to perform southern blot hybridization with a digoxigenin-labelled *fosA3*

DNA probe (Roche, Germany).

Conjugation experiment

Horizontal transmission ability was determined for all *fosA3* genes, and sodium azide-resistant *E. coli* J53 was used as the recipient for conjugation. The transconjugant was selected on a MacConkey agar plate supplemented with 150 mg/L sodium azide, 128 mg/L fosfomycin, and 25 mg/L glucose-6-phosphate. The transconjugant underwent PCR amplification and Sanger sequencing to confirm the transfer of the *fosA3* gene.

Whole-genome sequencing analysis

Total genomic DNA, extracted using a Hipure Bacterial DNA Kit (Magen, China), was subjected to whole-genome sequencing by Novogene (Beijing Novogene Bioinformatics Co., Ltd., China) using Illumina platform Novo-PE150 technology and to long-read sequencing using Oxford Nanopore MinION (Oxford Nanopore Technologies, UK). SPAdes v3.8.7 (Bankevich et al., 2012) was used for *de novo* assembly. Unicycler v0.4.7 (Wick et al., 2017) was used to obtain the assembled genome. Whole-genome sequencing data were analyzed *in silico* using MLST v2.11 (https://github.com/tseemann/mlst) for multi-locus sequence typing, ABRicate v0.8 (https://github.com/tseemann/abricate) for screening ARGs, plasmid types, and virulence factors, and SeroTypeFinder v2.0 for serotyping (Joensen et al., 2015). Sequence alignment was performed by Easyfig v2.1 (Sullivan et al., 2011). A phylogenetic tree based on core genome single nucleotide polymorphisms (cgSNPs) was constructed using Parsnp v1.5.4 (https://github.com/marbl/parsnp). Snippy v4.6.0 (https://github.com/tseemann/snippy) was used to calculate total SNP quantity.

Nucleotide sequence accession number

The assembled genomes of the *E. coli* isolates (XJW9B263 and XJW9B277) based on long-read sequencing were submitted to GenBank under accession Nos. CP067399–CP067401 and CP068041–CP068045, respectively. The raw reads (Illumina) of the *E. coli* isolates (XJW9B298, XJW9B290, XJW9B274, XJW9B277, XJW9B263, and XJW9B285) were deposited in the Genome Sequence Archive (GSA) under accession No. CRA004296.

RESULTS

Overall resistance phenotypes of *E. coli* isolates

A total of 33 non-duplicate *E. coli* strains were obtained. The antimicrobial susceptibility results showed that of the 33 *E. coli* isolates, 29 (87.9%) exhibited resistance to five or more antimicrobials, and most were resistant to critically important antimicrobials (CIAs), including third and fourth generation cephalosporins (*n*=24) and ciprofloxacin (*n*=24) (Figure 1). In particular, 16 (48.5%) isolates showed resistance to fosfomycin, as well as cephalosporins and ciprofloxacin.

Molecular characterization of *fosA3*-positive *E. coli*

PCR screening confirmed that all fosfomycin-resistant isolates were positive for *fosA3.* In total, 93.8% (*n*=15), 93.8% (*n*=15), and 25.0% (*n*=4) of *fosA3*-positive isolates co-harbored bla_{CTX-M}, *floR*, and *rmtB*, respectively, with bla_{CMY-2}, *armA*, and *mcr-1* not detected.

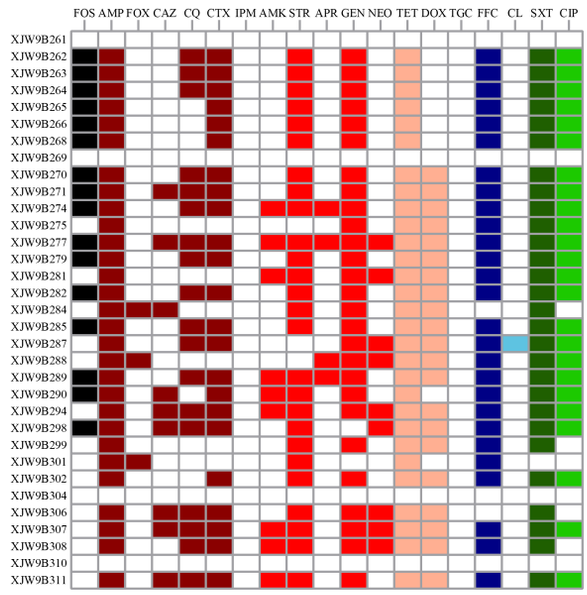

Figure 1 Antimicrobial resistance phenotypes of all *E. coli* isolates

FOS, fosfomycin; AMP, ampicillin; FOX, cefoxidine; CAZ, ceftazidime; CQ, cefquinome; CTX, cefotaxime; IPM, imipenem; AMK, amikacin; STR, streptomycin; APR, apramycin; GEN, gentamicin; NEO, neomycin; TET, tetracycline; DOX, doxycycline; TGC, tigecycline; FFC, florfenicol; CL, colistin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin.

PFGE was successfully performed for all *fosA3*-carrying isolates, and four different *Xba*I PFGE patterns (A to D) were observed (Figure 2). Pattern D, which included 11 (68.8%) *fosA3*-carrying isolates, was dominant, followed by pattern C (*n*=3), suggesting pandemic pattern D *fosA3*-carrying *E. coli* isolates in this cattle farm.

The conjugation results indicated that five *fosA3* genes in A-, B-, and C-pattern isolates were successfully transferred to *E. coli* J53. The S1-PFGE results confirmed that the *fosA3* genes in the A- and C-pattern isolates were located on single plasmids of the same size (~138.9 kb), and the *fosA3* gene in the B-pattern isolate was located on a single plasmid (~78.2 kb). Southern blot hybridization revealed that the other 11 *fosA3* genes that failed in the conjugation experiment were located on the same-size band (~1 135 kb) in all D-pattern isolates, indicating a chromosomal location for the *fosA3* gene.

The PCR mapping results demonstrated that all *fosA3* genes were flanked by IS*26*. In total, three common types of IS*26* composite transposons were found, including IS*26*-ΔIS*Ecp1*-$bla_{CTX-M-14}$-ΔIS*903*-*fosA3*-*orf1*-Δ*orf2*-IS*26* (*n*=4), IS*26*-*fosA3*-*orf1*-*orf2*-Δ*orf3*-IS*26* (*n*=1), and IS*26*-*fosA3*-*orf1*-Δ*orf2*-IS*26* (*n*=11) (Supplementary Figure S1).

Genomic analysis of *fosA3*-positive *E. coli*

Whole-genome sequencing was performed on six *fosA3*-harbouring *E. coli* isolates with four different PFGE patterns, and the obtained data were analyzed *in silico*. The sequence

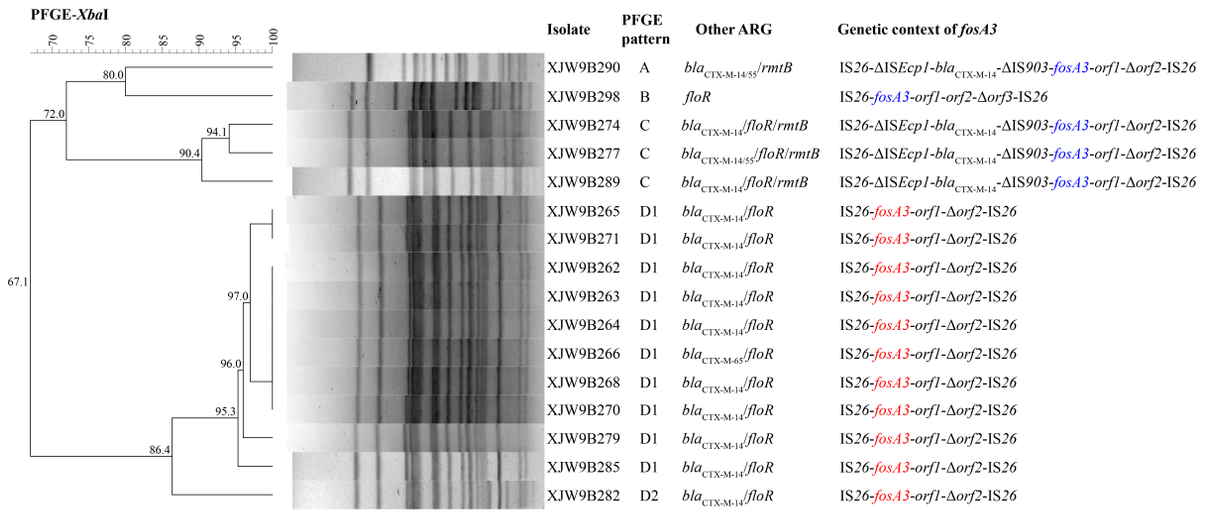

Figure 2 PFGE profiles, antimicrobial resistance genes, and genetic structure of *fosA3*-positive *E. coli*

fosA3 genes in blue and red indicate location on plasmid and chromosome, respectively.

type (ST) of the dominant D-pattern *fosA3*-harbouring *E. coli* isolates (XJW9B263 and XJW9B285) was ST10. The other isolates were ST167 (XJW9B274 and XJW9B277), ST1431 (XJW9B298), and unknown ST (XJW9B290). The O101:H9 serotype was obviously dominant among the six whole-genome sequencing isolates, except that the serotype of *E. coli* XJW9B298 (ST1431) was O8:H30. In addition, multiple ARGs were detected among all whole-genome sequencing isolates, three of which carried the F18:A-:B1 plasmid (Supplementary Table S2).

The D-pattern isolate XJW9B263 and C-pattern isolate XJW9B277 were further subjected to long-read sequencing to obtain assembled genomes. Sequence analysis confirmed that *fosA3* and $bla_{\text{CTX-M-14}}$ were co-located on the chromosome of XJW9B263 (GenBank: CP067399) and on the 133 299 bp long F18:A-:B1 plasmid pHNXJB277 of XJW9B277 (GenBank accession No.: CP068043).

NCBI-BLAST analysis revealed that pHNXJB277 showed genomic sequence identity with the *fosA3*-positive F18:A-:B1 plasmid pT28-2R of pet dog origin in Henan Province, China (GenBank accession No.: CP049355.1) (Figure 3A). The backbones of the two F18:A-:B1 plasmids were highly similar, while the multi-resistance region (MRR) varied. Similarly, *fosA3* genes co-localized with $bla_{\text{CTX-M-14}}$ on these two plasmids were surrounded by IS*26*.

Core genome SNP calling of *E. coli* ST10 and ST167

To explore the origin of pandemic *E. coli* ST10 and ST167, the assembled contigs of 88 164 *E. coli* isolates from GenBank were collected to identify STs. A total of 3 444 *E. coli* isolates of ST10 and 336 *E. coli* isolates of ST167 were detected and further subjected to cgSNP-based phylogenetic analysis with the pandemic *E. coli* ST10 (isolates XJW9B263 and XJW9B285) and *E. coli* ST167 (isolates XJW9B274 and XJW9B277) identified in this cattle farm. Based on our results, all *E. coli* ST167 isolates from GenBank showed >390 cgSNP differences from XJW9B277, whereas isolates XJW9B274 and XJW9B277 were clonal with only one cgSNP distance. We also calculated the number of cgSNPs in 45 *E. coli* ST10 isolates that were related to the XJW9B263 clone against the XJW9B263 reference isolate. Isolates XJW9B285 and XJW9B263 from the cattle farm were clonal with no cgSNP difference. However, most of the *E. coli* ST10 isolates from GenBank showed >100 cgSNP differences from XJW9B263, including isolates originating from humans and animals in China; only seven isolates derived from humans and animals outside China had <100 cgSNP differences from XJW9B263. Of note, an *E. coli* ST10 strain (GenBank: GCA_014156895.1) isolated from a rectal swab of an Australian silver gull (*Chroicocephalus*) in 2017 differed from isolate XJW9B263 by just 11 cgSNPs (Figure 4), suggesting a close relationship according to the recommended ≤10 SNP threshold of *E. coli* (Schürch et al., 2018).

Serotypes, ARGs, virulence genes, and plasmids were screened using whole-genome sequences of the original Australian silver gull *E. coli* isolate (GCA_014156895.1), and further compared with isolate XJW9B263. Results showed that both were *E. coli* O101:H9-ST10 and carried identical resistance genes ($bla_{\text{CTX-M-14}}$, $bla_{\text{TEM-1B}}$, *aph(3'')-Ib*, *aph(6)-Id*, *aac(3)-IId*, *tet*(A), *cmlA1*, *floR*, *mdf*(A), *mph*(A), *fosA3*, *sul2*, *dfrA14*) and plasmids (IncFIB and IncY) (Table 1). XJW9B263 was distinguished from GCA_014156895.1 by only four virulence-associated genes (*ecpD*, *entD*, *espL4*, and *espX4*), and GCA_014156895.1 additionally carried Col-like replicon Col(MG828). Moreover, the contigs of GCA_014156895.1 successfully matched a partial chromosome of XJW9B263 containing *fosA3*, $bla_{\text{CTX-M-14}}$, and other ARGs (Figure 3B)

DISCUSSION

With the widespread use of antimicrobials among humans and animals, MDR bacteria have emerged (Nikaido, 2009; Schürch et al., 2018), challenging the clinical treatment of bacterial infections. During routine surveillance of antimicrobial resistance in a cattle farm located in Xinjiang, a high prevalence of MDR *E. coli* isolates of diarrheal calf origin was observed. In particular, a surprisingly high fosfomycin resistance rate (48.5%) was noted, much higher than that of

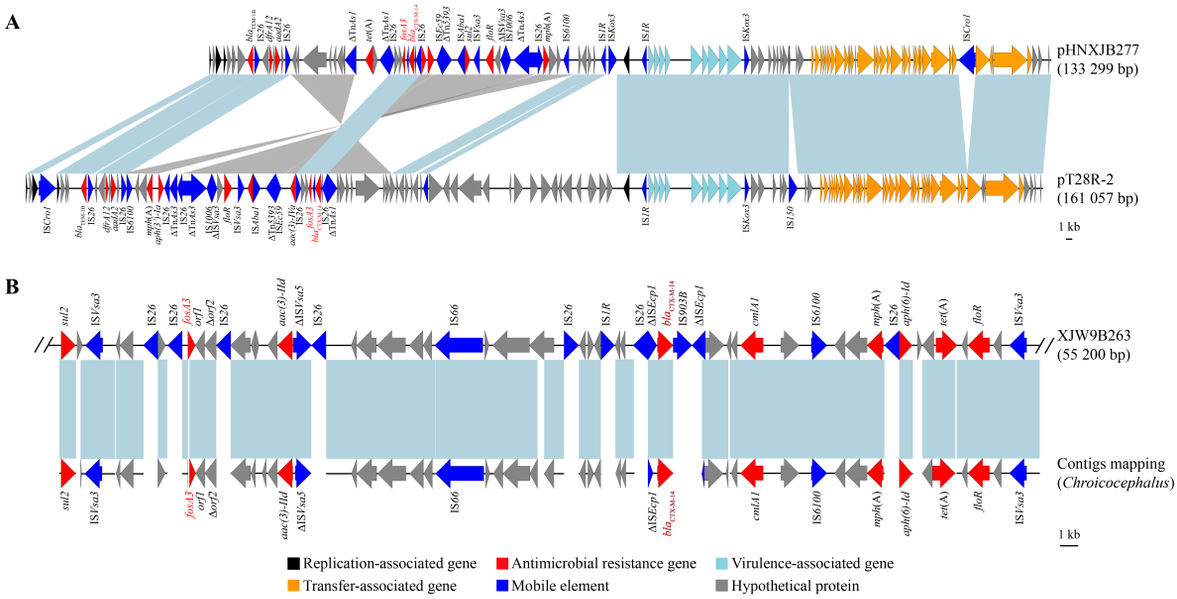

Figure 3 Genetic environment of *fosA3* and $bla_{CTX\text{-}M\text{-}14}$

A: Complete sequence comparison of two F18:A-:B1 plasmids (pHNXJB277, GenBank accession No.: CP068043; pT28R-2, GenBank accession No.: CP049355.1). B: Incomplete chromosomal sequence of XJW9B263 (GenBank accession No.: CP067399), containing same ARGs as *Chroicocephalus*-derived isolate (GenBank accession No.: GCA_014156895.1). Blue and gray shadows indicate homologous regions in same and opposite directions, respectively; same color arrows indicate same genes. Branch length is drawn to scale.

other food animals. For example, fosfomycin resistance rates in chicken-origin *E. coli* isolates from Guangdong and Northeast China are reported at 27.9% (He et al., 2017) and 27.4% (Jiang et al., 2017), respectively. PCR screening showed that *fosA3* genes were present in all fosfomycin-resistant isolates, and thus may mediate fosfomycin resistance. All FosA3-producers also showed resistance to cephalosporins and ciprofloxacin, and almost all *fosA3*-producers co-harbored $bla_{CTX\text{-}M}$, as commonly described nation- and worldwide (Cunha et al., 2017; Hou et al., 2012; Lupo et al., 2018; Lv et al., 2020; Yang et al., 2014). Regarding this, co-selection by long-term use of cephalosporins and enrofloxacin in this cattle farm may account for the prevalence of *fosA3*, as reported in a Chinese broiler farm, in which co-selection was considered the hypothetical driving force for the prevalence of plasmid-mediated colistin resistance gene *mcr-1* (Cao et al., 2020).

Previous studies have reported that the *fosA3* gene in *E. coli* is not generally spread by clonal transmission, but rather by plasmid-mediated horizontal transmission. Here, however, we found that most *fosA3*-positive isolates shared similar PFGE profiles, belonging to *E. coli* O101:H9-ST10 (*n*=2) and O101:H9-ST167 (*n*=2), indicating that the spread of *fosA3* genes in this cattle farm was likely mediated by vertical clonal transmission.

Serotype O101, which is associated with animal and human diseases, is frequently detected among pathogenic *E. coli* (Chirila et al., 2017; Mandal et al., 2001; Tan et al., 2012). To the best of our knowledge, however, serotype O101:H9 has only been reported in Shiga toxin-producing *E. coli* (STEC) from humans with diarrheal disease in Germany (Beutin et al., 2008), in enterotoxigenic *E. coli* (ETEC) from diarrheal calves in Europe (Contrepois et al., 1998) and children with diarrheal disease in New Caledonia (Begaud et al., 1993), and in *E. coli* isolated from humans with acute suppurative cholangitis (Sung et al., 1994). In view of the limited number of reports of serotype O101:H9 in China, core genomes of the O101:H9-ST10 and O101:H9-ST167 clones were compared with those of all *E. coli* isolates submitted in GenBank to explore the origin of the *E. coli* clones in this cattle farm. Results demonstrated that all GenBank *E. coli* ST167 isolates were clonally unrelated to the O101:H9-ST167 clone (isolates XJW9B274 and XJW9B277) detected in this study, while core genomes of 45 *E. coli* ST10 isolates from GenBank were relatively similar to those of the O101:H9-ST10 clone (isolates XJW9B263 and XJW9B285). Further analysis confirmed that most showed >100 cgSNP differences from the O101:H9-ST10 clone; nevertheless, six isolates, all isolated from humans outside of China, including Canada and several European countries (UK, Germany, France, and Estonia), differed from the O101:H9-ST10 clone by <100 cgSNPs. Surprisingly, the core genome sequence of an Australian *Chroicocephalus*-derived *E. coli* isolate showed high similarity to the O101:H9-ST10 clone, with just 11 cgSNP differences, and the ARG profiles and plasmid types of both were very similar, indicating a significant clonal relationship. We note that wild birds forage for food at this cattle farm throughout the year, and that cattle feed is often contaminated by bird droppings (Supplementary Figure S2). Considering that the core genome of this clone exhibits greater similarity to foreign isolates, we suspect that the *E. coli* O101:H9-ST10 clone spreading in this cattle farm originated from foreign wild birds, i.e., the *Chroicocephalus*-bearing *E. coli* O101:H9-ST10. In accordance with a migration map of waterbirds worldwide

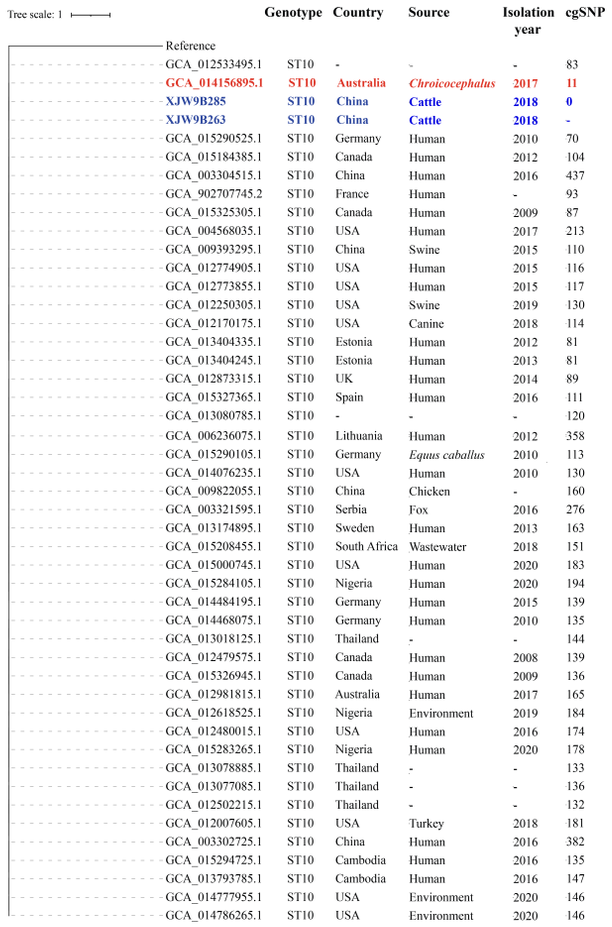

Figure 4 Core genome SNP-based phylogenetic tree of *E. coli* ST10 strains

cgSNP indicates total amount of core genome SNPs in *E. coli* ST10 strains against reference isolate XJW9B263.

(https://www.eaaflyway.net/), gulls and terns annually traverse the East Asian-Australasian Flyway (EAAF) covering East Asian countries and Australia. Furthermore, Australian *Chroicocephalus* often mix with the great crested terns (*Thalasseus bergii cristatus*) that fly to Australia in the south and to Ryukyu Islands and southeastern China in the north (https://birdsoftheworld.org/bow/home). Therefore, although the Australian *Chroicocephalus* does not migrate to Xinjiang, wild birds foraging for food at the cattle farm may mix with the great crested terns that show an overlapping distribution with the Australian *Chroicocephalus*, from where they acquire the *E. coli* O101:H9-ST10 clone (Figure 5).

Furthermore, though the estimated number of core genome SNPs per year for *E. coli* is unclear, a cutoff of ≤21 SNPs per genome per year for *Klebsiella pneumoniae* and a ≤23 SNP threshold for *Enterobacteriales* of local transmission have been reported (David et al., 2019; Sherry et al., 2019). Therefore, the 11 cgSNP distance between the two *E. coli* O101:H9-ST10 strains derived from *Chroicocephalus* and cattle suggests short-term clonal transmission of the high-risk *E. coli* ST10 from migratory *Chroicocephalus* birds to the calves.

Although wild birds (gulls) are not exposed to antimicrobials directly, their coastal habitats result in high-level human contact. As such, these birds have been described as reservoirs and vectors of MDR bacteria for the global diffusion of ARGs mediating resistance to CIAs (Mukerji et al., 2019, 2020; Villa et al., 2015; Wang et al., 2017a). To date, *Chroicocephalus* birds have not been reported in Yili in Xinjiang; however, indirect transmission of MDR bacteria from *Chroicocephalus* to wild birds that visit this cattle farm is possible. Due to their outdoor breeding, calves may potentially acquire MDR bacteria spread by wild birds that forage or fly over the farm. Thus, greater attention should be paid to wild birds visiting farms and sanitation should be strengthened to slow the potential risk of migratory bird dissemination of MDR bacteria.

CONCLUSIONS

This study described the clonal spread of FosA3- and CTX-M-producing *E. coli* O101:H9-ST10 among diarrheal calves from a cattle farm in Xinjiang, China. We speculate that the clones originated from migratory (foreign) birds and were transmitted by wild birds foraging on the farm. This is the first direct evidence of migratory birds disseminating bacteria resistant to CIAs across land and countries. These results highlight the

Table 1 Genomic analysis of clonal *E. coli* O101:H9-ST10

Isolate	XJW9B263	GCA_014156895.1
Collection year	2018	2017
Country	China	Australia
Source	Calf	*Chroicocephalus*
Serotype	O101:H9	O101:H9
Antimicrobial resistance gene	*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$, *aph(3'')-Ib*, *aph(6)-Id*, *aac(3)-IId*, *tet*(A), *cmlA1*, *floR*, *mdf*(A), *mph*(A), *fosA3*, *sul2*, *dfrA14*	*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$, *aph(3'')-Ib*, *aph(6)-Id*, *aac(3)-IId*, *tet*(A), *cmlA1*, *floR*, *mdf*(A), *mph*(A), *fosA3*[a], *sul2*, *dfrA14*
Virulence gene	*aslA*, *ecpA*, *ecpB*, *ecpC*, *ecpD*, *ecpE*, *ecpR*, *entA*, *entB*, *entC*, *entD*, *entE*, *entF*, *entS*, *espL1*, *espL4*, *espX1*, *espX4*, *espX5*, *espY1*, *fdeC*, *fepA*, *fepB*, *fepC*, *fepD*, *fepG*, *fes*, *fimB*, *fimC*, *fimD*, *fimE*, *fimF*, *fimG*, *fimH*, *fimI*, *ompA*	*aslA*, *ecpA*, *ecpB*, *ecpC*, *ecpE*, *ecpR*, *entA*, *entB*, *entC*, *entE*, *entF*, *entS*, *espL1*, *espX1*, *espX5*, *espY1*, *fdeC*, *fepA*, *fepB*, *fepC*, *fepD*, *fepG*, *fes*, *fimB*, *fimC*, *fimD*, *fimE*, *fimF*, *fimG*, *fimH*, *fimI*, *ompA*
Plasmid	IncFIB, IncY	IncFIB, IncY, Col(MG828)

[a] indicates delta *fosA3* gene likely truncated by whole-genome sequencing. Virulence genes and plasmids that differ from each other are underlined.

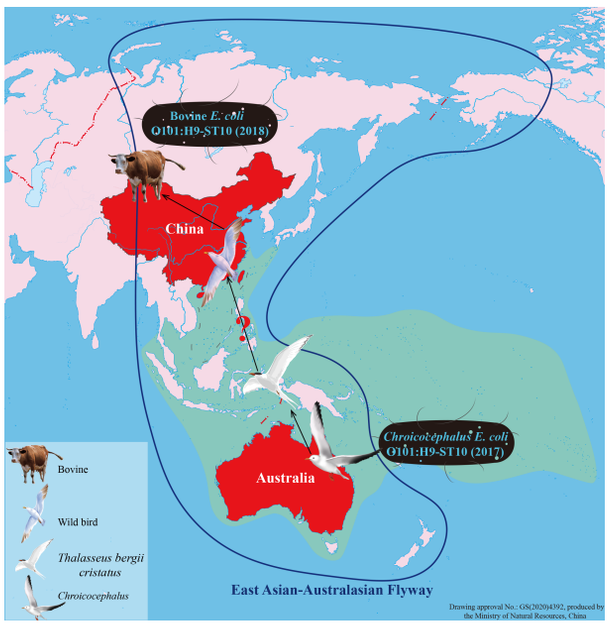

Figure 5 Schematic of possible global dissemination of *E. coli* O101:H9-ST10 from Australian *Chroicocephalus* to Chinese cattle
Green shadow indicates distribution of great crested tern (*Thalasseus bergii cristatus*).

need to pay greater attention to the risk of migratory birds spreading MDR microorganisms on a global scale. Moreover, biosafety prevention and control should not only focus on terrestrial pathogen contact, but also pathogens disseminated by birds and/or insects during aerial flight.

DATA AVAILABILITY

The datasets in this study can be found in GenBank under accession Nos. CP067399-CP067401 (XJW9B263) and CP068041-CP068045 (XJW9B277), and in the GSA databank under accession No. CRA004296 (XJW9B298, XJW9B290, XJW9B274, XJW9B277, XJW9B263, and XJW9B285).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.H.L. and J.Y. conceived the research. W.Y.H., X.X.Z., J.Y., G.L.G., M.Y.G., Z.P.C., and L.C.L. collected the data. J.H.L., W.Y.H., X.X.Z., J.Y., L.C.L., F.G.Z., and X.F.S. analyzed and interpreted the data. W.Y.H. drafted the manuscript, J.H.L., J.Y., and X.F.S. revised the report. All authors read and approved the final version of the manuscript.

ACKNOWLEDGEMENTS

We are grateful to Xiao-Jun Yang from the Kunming Institute of Zoology, Chinese Academy of Sciences, for helpful comments on this study.

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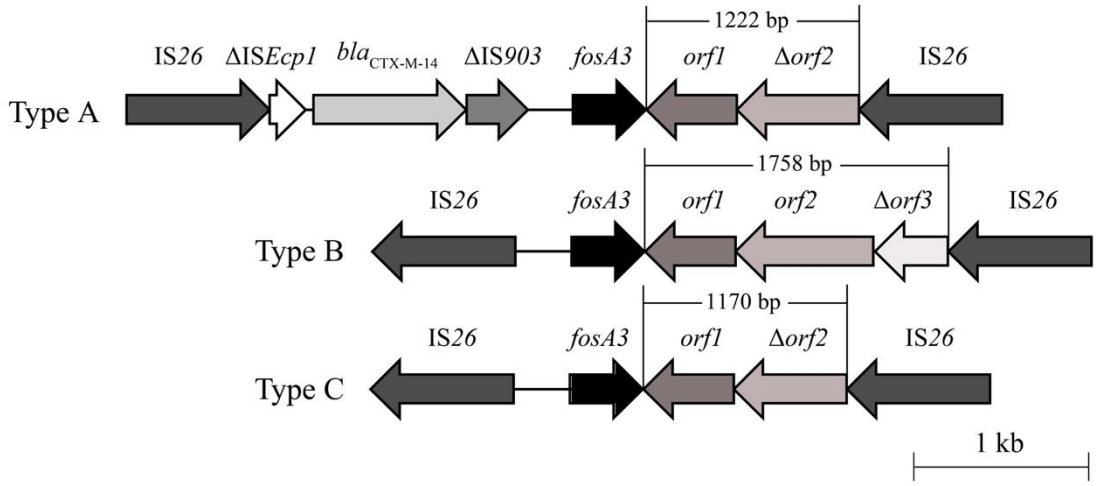
Supplementary Materials

Supplementary Table S1 Primers used for PCR in this study

Target	Primer name	Sequence (5'-3')	Size (bp)
fosA3	*fosA3*-F	GCGTCAAGCCTGGCATTT	282
	fosA3-R	GCCGTCAGGGTCGAGAAA	
$bla_{CTX-M-1G}$	$bla_{CTX-M-1G}$-F	CTTCCAGAATAAGGAATCCC	949
	$bla_{CTX-M-1G}$-R	CGTCTAAGGCGATAAACAAA	
$bla_{CTX-M-9G}$	$bla_{CTX-M-9G}$-F	TGACCGTATTGGGAGTTTG	902
	$bla_{CTX-M-9G}$-R	ACCAGTTACAGCCCTTCG	
bla_{CMY-2}	bla_{CMY-2}-F	CACTCAAGGATGTATTGTG	1143
	bla_{CMY-2}-R	TTAGCGTTGCCAGTGCTCG	
armA	*armA*-F	CAATCAGGGGCAGTTATCA	529
	armA-R	CCCTATAACCTTCGAATC	
rmtB	*rmtB*-F	ACATCAACGATGCCCTCAC	725
	rmtB-R	AAGTTCTGTTCCGATGGTC	
mcr-1	*mcr-1*-F	CGGTCAGTCCGTTTGTTC	309
	mcr-1-R	CTTGGTCGGTCTGTAGGG	
floR	*floR*-F	CTGAGGGTGTCGTCATCTAC	673
	floR-R	GCTCCGACAATGCTGACTAT	
IS*26*-*fosA3*	IS*26*-*fosA3*-F	GCACGCATCACCTCAATACC	Unknown
	IS*26*-*fosA3*-R	TCATCCAGCGACAAGCACA	
fosA3-IS*26*	*fosA3*-IS*26*-F	GGGGCTGAGGTATGGAAAGA	Unknown
	fosA3-IS*26*-R	AGGAGATGCTGGCTGAACG	

Supplementary Table S2 Genomic analysis of *fosA3*-positive *E. coli*

Isolate	Sequence type	Serotype	Antimicrobial resistance gene	Plasmid
XJW9B290	New	O101:H9	*bla*$_{CTX-M-14}$, *bla*$_{CTX-M-55}$, *aadA2*, *aph(3')-IIa*, *aph(3'')-Ib*, *aph(4)-Ia*, *aph(6)-Id*, *aac(3)-IVa*, *rmtB*, *mdf*(A), *mph*(A), *fosA3*, *sul2*, *dfrA12*, *qepA1*	F18:A-:B1, IncI1, IncX1
XJW9B298	ST1431	O8:H30	*ant(3'')-Ia*, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(6)-Id*, *tet*(A), *floR*, *lnu*(F), *mdf*(A), *fosA3*, *sul2*, *sul3*, *dfrA14*, *qnrS1*, *ARR-3*	F40:A-:B24, IncHI2, IncN1, IncY
XJW9B274	ST167	O101:H9	*bla*$_{CTX-M-14}$, *aadA2*, *aph(3'')-Ib*, *aph(4)-Ia*, *aph(6)-Id*, *aac(3)-IVa*, *rmtB*, *tet*(A), *floR*, *mdf*(A), *mph*(A), *fosA3*, *sul2*, *dfrA12*, *qepA1*	F18:A-:B1, IncI1
XJW9B277	ST167	O101:H9	*bla*$_{CTX-M-14}$, *bla*$_{CTX-M-55}$, *bla*$_{TEM-1B}$, *aadA2*, *aadA22*, *ant(3'')-Ia*, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(4)-Ia*, *aph(6)-Id*, *aac(3)-IId*, *aac(3)-IVa*, *rmtB*, *tet*(A), *floR*,	F18:A-:B1, IncI1, IncHI2-IncN1
XJW9B263	ST10	O101:H9	*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$, *aph(3'')-Ib*, *aph(6)-Id*, *aac(3)-IId*, *tet*(A), *cmlA1*, *floR*, *mdf*(A), *mph*(A), *fosA3*, *sul2*, *dfrA14*	IncFIB, IncY
XJW9B285	ST10	O101:H9	*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$, *aph(3'')-Ib*, *aac(3)-IId*, *tet*(A), *cmlA1*, *floR*, *mdf*(A), *mph*(A), *fosA3*, *sul2*, *dfrA14*	IncFIB, IncY



Supplementary Figure S1 Schematic of genetic context of *fosA3* gene

Branch length is drawn to scale. Spacer regions between 3' end of *fosA3* and IS*26* are 1 222 bp, 1 758 bp, and 1 170 bp, respectively.

Supplementary Figure S2 Wild birds (A) and their droppings (B) in cattle farm