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Analysis of antimicrobial resistance and genetic correlations of *Escherichia coli* in dairy cow mastitis

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Abstract

Introduction: *Escherichia coli* is a widespread environmental pathogen frequently causing dairy cow mastitis. This bacterium is particularly capable of acquiring antimicrobial resistance, which can have severe impacts on animal food safety and human health. The objective of the study was to investigate antimicrobial resistance and genetic correlations of *E. coli* from dairy cow mastitis cases in northern China. **Material and Methods:** Forty strains of *E. coli* from 196 mastitis milk samples were collected, susceptibility to 13 common antibiotics and the prevalence of resistance genes were tested in these strains, and the genetic characteristics were identified by multilocus sequence typing. **Results:** The results showed that most isolates were multidrug resistant (MDR) (75%), and the resistance rates to cefazolin, trimethoprim-sulfamethoxazole and ampicillin were 77.5%, 55.0%, and 52.5%, respectively. The representative genes of the isolates were *aadA* (62.5%) and *tet*(B) (60.0%). Multilocus sequence typing showed 19 different sequence types (STs) and 5 clonal complexes (CCs) in the 40 isolates, mainly represented by ST10 and CC10. The strains of the same ST or CC showed a high level of genetic relatedness, but the characteristics of their antimicrobial resistance were markedly different. **Conclusion:** Most *E. coli* isolates in the study were MDR strains. Some strains of the same ST or CC showed diverse resistance characteristics to common antimicrobials. Therefore, *E. coli* from dairy cow mastitis in northern China should be investigated to elucidate its antimicrobial resistance and genotypes.

Keywords: *Escherichia coli*, molecular characteristics, antimicrobial resistance, genetic correlation, dairy cow mastitis.

Introduction

Dairy cow mastitis is a disease that is common on more than one continent and causes a decline in milk production and quality, leading to significant economic losses in the dairy industry (36). *Escherichia coli* is an important mammary pathogen in the environment of dairy farms and is closely associated with severe inflammatory symptoms (15). Currently, antibiotics are widely used to prevent and treat dairy cow mastitis. In the United States of America, it has been estimated that more than 80% of the total administered volume of antibiotics was used in animal production activities (14). Although the usage of antimicrobials usually brings positive effects, the problem of antimicrobial resistance (AMR) has also emerged (18). A nationwide study was conducted in China to determine the extent of AMR in common mastitis pathogens (including *E. coli*) infecting dairy herds and the study showed its increasing prevalence (9). Antimicrobial resistance reduced the cure rates of cow mastitis and posed a grave threat to public health and animal welfare (42).

Antimicrobial-resistant bacteria often harbour antibiotic resistance genes, which is proven to be a driving factor in drug resistance (39). These bacteria can spread among different hosts, which transduce antibiotic resistance genes to strains which may in some cases already possess certain drug resistance, leading to the emergence of multi-drug-resistant (MDR) bacteria (35).

As a clonally structured population, *E. coli* was classified into different phylogenetic groups and clonal complexes (CCs) by multilocus sequence typing (MLST) (23). By comparing database sequence records with the MLST results, specific housekeeping genes of *E. coli* were indicated to denote different STs; such a comparison was considered a reliable molecular typing

method to explore the genetic correlations of microbial populations (24). Recent epidemiological investigations suggested that the antibiotic resistance of *E. coli* strains exhibited diversity in different microbial populations. It also brought more challenges to the prevention and treatment of dairy cow mastitis in veterinary clinics (32). In the face of the growing problem of antibiotic resistance and the potential threat to human health, it is necessary to analyse the antibiotic resistance of different *E. coli* populations. The objectives of the study were to understand the current state of antimicrobial resistance and the genetic characteristics of *E. coli* strains from dairy cow mastitis and to provide information helpful for the rational use of antibiotics in clinics.

Material and Methods

Sample collection and *E. coli* isolation. Between June 2019 and October 2021, 196 mastitis milk samples were collected from different dairy farms located in Xushui, Qingyuan, Quyang, and Mancheng in northern China. The criteria defining clinical mastitis were local pain in the mammary gland area accompanied by severe or general signs of inflammation, including swelling of the udder, tenderness to touch, fever, and depression (30, 40). Following convention, the teat was disinfected with 2% iodine tincture and 75% ethanol and the three initial streams were forestripped. Milk samples were aseptically collected in sterile tubes immediately. The samples were put on ice and sent to the laboratory within 4 h. A 10 µL volume of milk was aerobically cultured at 37°C for 12 h in blood agar (Aobox, Beijing, China) with 5% sheep blood. Primary identification of the *E. coli* isolates was based on the characteristics of a Gram stain and growth on Eosin-Methylene Blue (EMB) Agar (Solarbio, Beijing, China). The 16S rDNA of all isolates was amplified in a PCR using 27F (5′-AGAGTT TGATCMTGGCTCAG-3′) and 1492R (5′-TACGGY TACCTTGTTACGACTT-3′) universal primers (37). The reaction procedures were as follows: 300 s at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 57°C and 60 s at 72°C. The PCR products were sequenced by Shanghai Sangon Biotech Co., Ltd (China) and compared with the sequences logged in GenBank. The confirmed *E. coli* isolates were stored in 25% sterile glycerol at −80°C.

Antimicrobial susceptibility test of *E. coli* isolates. Susceptibility to antimicrobial agents was determined by the Kirby–Bauer method as described by the Clinical Laboratory Standards Institute (CLSI) guidelines (11). Confirmed isolates of *E. coli* were tested for susceptibility to 13 antimicrobial agents commonly used in China. The preparations included beta-lactams, aminoglycosides, macrolides, tetracyclines, sulfonamides and quinolones; the selection was ampicillin (AMP, 10 µg), amoxicillin (AMX, 10 µg), ceftriaxone (CRO, 30 µg), cefazolin (CFZ, 30 µg), gentamicin (GEN, 10 µg), streptomycin (STR, 10 µg), neomycin (NER, 30 µg), amikacin (AMI, 30 µg), erythromycin (EM, 15 µg), doxycycline (DOX, 30 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), ciprofloxacin (CIP, 5 µg) and enrofloxacin (ENR, 5 µg). The antimicrobial agents were purchased from the China Institute of Veterinary Drugs Control. Multidrug resistance (MDR) was defined as resistance to three or more classes of antibiotics. *Escherichia coli* ATCC 25922 was used as a reference strain.

DNA extraction. A single colony from a fresh bacterial culture on EMB Agar was picked and inoculated into 5 mL of fresh Luria–Bertani broth and incubated with shaking for 12 h. Extraction of DNA was achieved using the DNA Quick extraction kit (Tiangen, Beijing, China) following the manufacturer's instructions. All DNA preparation concentrations were measured using a Nanodrop ND-2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and adjusted to be approximately 100 ng/mL. The DNA extracts were stored at −20°C.

MLST and phylogenetic group. One pair of primers for each of the *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* housekeeping genes was designed utilising data from a public MLST database (https://pubmlst.org/data) and then used in a PCR (43). The reaction procedure was as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at different temperatures for 45 s (Table 1), extension at 72°C for 60 s, and final extension at 72°C for 7 min. The products were sequenced by Shanghai Sangon Biotech Co., Ltd. The sequences of housekeeping genes were processed by BioEdit (https://www.bioedit.com) to obtain the housekeeping gene number and they were submitted to the Achtman online database (http://enterobase.warwick.ac.uk/species/index/ecoli) for comparison. Each isolate's ST was acquired from the database. A minimum spanning tree was built using the goeBURST algorithm in Phyloviz1.0 software (http://www.phyloviz.net) (38). Subsequently, the sequences were further trimmed and concatenated (3,370 bp) to conduct molecular phylogenetic analysis using the maximum likelihood method in MEGA 7.0. Bootstrapping with 1,000 replicates was applied to estimate the reliability of the phylogenetic tree. The tree was visualised with iTOL online software (https://itol.embl.de) to analyse the distribution of drug resistance genes and resistance phenotype in the *E. coli* isolates.

Detection of antimicrobial resistance genes. Genes were detected by PCR with resistance to the following antimicrobials: beta-lactams (*blaTEM*, *blaSHV* and *blaOXA*), aminoglycosides (*aac*(2'), *aacA4* and *aadA*), macrolides (*erm*(B) and *erm*(C)), tetracyclines (*tet*(A) and *tet*(B)), sulfonamides (*sul1* and *sul2*) and quinolones (*qnrB*) (1, 2, 28, 44). The reaction procedure of PCR was as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at different temperatures for 30 s (Table 1), extension at 72°C for 30 s, and final extension at 72°C for 10 min. PCR products were visualised by 1% agarose gel electrophoresis.

Statistical analysis. The chi-squared test and Pearson's correlation coefficient were used to compare the correlations between an isolate's resistance to a particular antibiotic and the isolate's possession of the corresponding resistance gene. The significance level was set at $P < 0.05$ for statistical procedures. All analyses were conducted using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Isolates. Overall, 40 (20.41%) *E. coli* isolates from the 196 tested milk samples were culture-positive. The isolation rates of *E. coli* in Xushui, Qingyuan, Quyang and Mancheng were 22.22% (16/72), 24.14% (7/29), 17.86% (5/28) and 17.91% (12/67), respectively.

Antimicrobial susceptibility testing. The results of the susceptibility test were interpreted according to the criteria of the CLSI (Table 2). Antimicrobial susceptibility tests showed that most of the isolates were resistant to CFZ (77.5%), SXT (55.0%) and AMP (52.5%). In contrast, most isolates were susceptible to AMI (95.0%), CIP (82.5%) and GEN (67.5%). In addition, some *E. coli* isolates were classified as intermediate susceptible to EM (52.5%), NER (37.5%), and ENR (27.5%) (Table 2). For analysis, intermediate susceptibility was considered as resistance (7). In this study, 29 (72.5%) MDR *E. coli* strains were detected. The most common antimicrobial resistance profile was AMP-AMX-CRO-CFZ-GEN-STR-NER-EM-DOX-SXT-CIP-ENR (n = 4, 13.8%). Resistance to eight antibacterial drugs was the most common occurrence of multiple resistance (n = 6, 20.7%) (Table 3).

Antimicrobial resistance genes. In the present study, 40 strains (100.0%) of *E. coli* with resistance genes were detected, and the genes detected most frequently were the aminoglycoside resistance gene, *aadA* (n = 25, 62.5%), the tetracycline resistance genes, *tet*(B) (n = 24, 60.0%) and *tet*(A), (n = 18, 45.0%) and the macrolide resistance gene, *erm*(B) (n = 16, 40.0%). Resistance genes to beta-lactams or macrolides, *blaTEM* and *erm*(C), were not detected in this study (Table 3).

Table 1. Primer sequences, product sizes, annealing temperature and references used for the PCR in the study

Gene	Primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference or GenBank accession no.
blaTEM	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	643	53	(25)
blaSHV	TTTGTCGCTTCTTTACTCGCCTTTA GCCAGATCCATTTCTATCATGCCTA	198	56	DQ247972
blaOXA	TCAACTTTCAAGATCGCA GTGTGTTTAGAATGGTGA	591	53	(25)
aac(2')	ACTGTGATGGGATACGCGTC CTCCGTCAGCGTTTCAGCTA	482	54	(26)
aacA4	CTTCAGGATGGCAAGTTGGT TCATCTCGTTCTCCGCTCAT	286	55	(26)
aadA	CTGGAGGTCACTGTCGTGC CCGTGGATTGCCAAAGGTC	274	55	X68089
erm(B)	AAAACTTACCCGCCATACCA TTTGGCGTGTTTCATTGCTT	126	53	MN461246
erm(C)	GCTCGTGTCATTTCTGGGAGT AGCCTAGCAGCCATTTCTATC	375	53	GQ483470
tet(A)	CGGAGCAGAAACAAGAAAGCG GGATCAGGACCGGATACACCAT	345	57	(26)
tet(B)	CATTAATAGGCGCATCGCTG TGAAGGTCATCGATAGCAGG	391	53	(26)
sul1	GCCTGGAACTGCTGCTGATGC TCGCCTGCCAAACCGAACTCT	314	59	(27)
sul2	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	793	57	(27)
qnrB	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC	513	55	(25)
adk	ATTCTGCTTGGCGCTCCGGG CCGTCAACTTTCGCGTATTT	583	54	(20)
fumC	TCACAGGTCGCCAGCGCTTC GTACGCAGCGAAAAAGATTC	806	54	(20)
gyrB	TCGGCGACACGGATGACGGC ATCAGGCCTTCACGCGCATC	911	60	(20)
icd	ATGGAAAGTAAAGTAGTTGTTCCGGCACA GGACGCAGCAGGATCTGTT	878	54	(20)
mdh	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	932	60	(20)
purA	CGCGCTGATGAAAGAGATGA CATACGGTAAGCCACGCAGA	816	54	(20)
recA	CGCATTCGCTTTACCCTGACC TCGTCGAAATCTACGGACCGGA	780	58	(20)

Table 2. Susceptibility of 40 *E. coli* strains to 13 antibiotics commonly used in China

Antibiotic	Distribution of *E. coli* strains (number of strains/%)			Decision criteria/Diameter of inhibitory zone (mm)		
	R	I	S	R	I	S
Ampicillin	21/52.5%	6/15.0%	13/32.5%	≤13	14–16	≥17
Amoxicillin	18/45.0%	3/7.5%	19/47.5%	≤13	14–17	≥18
Ceftriaxone	19/47.5%	1/2.5%	20/50.0%	≤19	20–23	≥24
Cefazolin	31/77.5%	8/20.0%	1/2.5%	≤19	20–22	≥23
Gentamicin	13/32.5%	0	27/67.5%	≤12	13–14	≥15
Streptomycin	12/30.0%	5/12.5%	23/57.5%	≤11	12–14	≥15
Neomycin	1/2.5%	15/37.5%	24/60.0%	≤11	12–16	≥17
Amikacin	1/2.5%	1/2.5%	38/95.0%	≤14	15–16	≥17
Erythromycin	17/42.5%	21/52.5%	2/5.0%	≤13	14–22	≥23
Doxycycline	14/35.0%	5/12.5%	21/52.5%	≤10	11–13	≥14
Trimethoprim-sulfamethoxazole	22/55.0%	0	18/45.0%	≤12	13–16	≥17
Ciprofloxacin	7/17.5%	0	33/82.5%	≤15	16–20	≥21
Enrofloxacin	6/5.0%	11/27.5%	23/57.5%	≤15	16–23	≥24

R – resistant; I – intermediate; S – susceptible

Table 3. Sequence types, resistance phenotypes and resistance genes in 40 *E. coli* strains

ID	Location	MLST	Resistance phenotypes	Resistance genes
1	Xushui	ST10	AMP-AMX-CRO-CFZ-GEN-STR-EM-DOX-SXT-ENR	*aadA, qnrB*
2	Xushui	ST10	AMP-AMX-CRO-CFZ-GEN-STR-NER-EM-DOX-SXT-CIP-ENR	*aadA, tet*(B)
3	Xushui	ST359	AMP-AMX-CRO-CFZ-GEN-STR-NER-EM-DOX-SXT-CIP-ENR	*blaSHV, aadA*
4	Xushui	ST10	CFZ-EM	*aadA, tet*(B), *sul2*
5	Xushui	ST10	AMP-AMX-CRO-CFZ-STR-NER-EM-DOX-SXT-CIP-ENR	*aadA, sul2*
6	Xushui	ST1585	AMP-AMX-CRO-CFZ-GEN-STR-NER-EM-DOX-SXT-CIP-ENR	*aacA4, aadA*
7	Xushui	ST359	AMP-AMX-CRO-CFZ-GEN-STR-NER-AMI-EM-DOX-SXT-CIP-ENR	*blaSHV, blaOXA, aadA*
8	Xushui	ST359	AMP-AMX-CRO-CFZ-GEN-NER-EM-DOX-SXT-ENR	*blaOXA, aacA4, aadA*
9	Xushui	ST359	AMP-AMX-CRO-CFZ-GEN-STR-EM-DOX-SXT-CIP-ENR	*blaSHV, blaOXA, aadA, tet*(B)
10	Xushui	ST10	CFZ-EM	*aadA, sul2*
11	Xushui	ST10	CFZ-EM	*aadA, tet*(B), *sul2*
12	Xushui	ST359	AMP-AMX-CRO-CFZ-GEN-NER-EM-SXT-ENR	*blaOXA, aadA, sul2*
13	Xushui	ST1125	AMP-AMX-CRO-CFZ-STR-SXT	*aadA, tet*(B)
14	Xushui	ST1585	AMP-AMX-CRO-CFZ-EM-DOX-SXT-ENR	*blaSHV, aadA, tet*(B)
15	Xushui	ST327	AMP-AMX-CRO-CFZ-EM-DOX-ENR	*aadA, tet*(B)
16	Xushui	ST937	AMP-AMX-CRO-CFZ-STR-EM-SXT-ENR	*aadA, tet*(B)
17	Qingyuan	ST10717	AMP-CFZ-STR-NER-EM-DOX-SXT	*aac*(2′), *aadA, erm*(B), *tet*(A), *tet*(B)
18	Qingyuan	ST942	CFZ-NER-EM-DOX	*erm*(B), *tet*(A), *sul1, sul2*
19	Qingyuan	ST446	AMP-CFZ-GEN-STR-NER-EM-DOX-SXT	*aac*(2′), *aadA, erm*(B), *tet*(A), *tet*(B)
20	Qingyuan	ST1310	AMP-AMX-CRO-CFZ-GEN-STR-NER-AMI-EM	*aac*(2′), *erm*(B), *tet*(A), *tet*(B), *sul2*
21	Qingyuan	ST515	AMP-AMX-CRO-CFZ-STR-EM-DOX-SXT-ENR	*aac*(2′), *erm*(B), *tet*(A), *sul1*
22	Qingyuan	ST48	AMP-CFZ-NER-EM	*aac*(2′), *erm*(B), *tet*(A), *tet*(B), *sul1*
23	Qingyuan	ST10	CFZ-NER-EM	*aac*(2′), *tet*(A), *sul2*
24	Quyang	ST1252	AMP-AMX-CRO-CFZ-STR-EM-SXT-ENR	*aac*(2′), *erm*(B), *tet*(A), *tet*(B)
25	Quyang	ST1079	AMP-CFZ-EM	*tet*(A), *tet*(B)
26	Quyang	ST154	CFZ	*aac*(2′), *erm*(B), *tet*(A), *tet*(B), *sul2*
27	Quyang	ST1585	AMP-AMX-CRO-CFZ-GEN-STR-NER-EM-DOX-SXT-CIP-ENR	*aadA, erm*(B)
28	Quyang	ST1167	AMP-AMX-CRO-CFZ-STR-NER-EM-DOX-SXT	*aadA, erm*(B), *tet*(A)
29	Mancheng	ST1610	AMP-CFZ-EM	*aac*(2′), *tet*(A)
30	Mancheng	ST10	NER-EM	*aac*(2′), *tet*(A), *sul1, sul2*
31	Mancheng	ST2741	CFZ-EM	*aac*(2′), *aadA, tet*(A), *tet*(B)
32	Mancheng	ST2741	CFZ-EM	*aac*(2′), *tet*(A), *tet*(B)
33	Mancheng	ST48	AMP-CFZ-GEN-STR-EM-DOX-SXT-ENR	*aac*(2′), *aadA, erm*(B), *tet*(A), *tet*(B), *sul1*
34	Mancheng	ST10	AMP-AMX-CRO-CFZ-EM-DOX-SXT-ENR	*aac*(2′), *erm*(B), *tet*(A), *tet*(B), *sul1, sul2*
35	Mancheng	ST906	AMP-AMX-CRO-CFZ-EM-SXT	*aac*(2′), *tet*(A), *tet*(B)
36	Mancheng	ST48	CFZ-EM	*aac*(2′), *aadA, erm*(B), *tet*(B), *sul1, qnrB*
37	Mancheng	ST48	CFZ-EM	*erm*(B), *tet*(B), *sul1*
38	Mancheng	ST48	CFZ-EM	*aadA, erm*(B), *tet*(B), *sul1*
39	Mancheng	ST906	CFZ-EM	*aac*(2′), *tet*(B)
40	Mancheng	ST48	AMP-AMX-CFZ-GEN-EM-DOX-SXT	*aadA, erm*(B), *sul1*

AMP – ampicillin; AMX – amoxicillin; CRO – ceftriaxone; CFZ – cefazolin; GEN – gentamicin; STR – streptomycin; NER – neomycin; AMI – amikacin; EM – erythromycin; DOX – doxycycline; SXT – trimethoprim-sulfamethoxazole; CIP – ciprofloxacin; ENR – enrofloxacin

Table 4. Pearson's correlation coefficients (r) of resistance genes and corresponding antibiotics

Resistance gene	Antimicrobials												
	AMP	AMX	CRO	CFZ	GEN	STR	NER	AMI	EM	DOX	SXT	CIP	ENR
blaSHV	0.231	0.317	0.333	0.053	-	-	-	-	-	-	-	-	-
blaOXA	−0.114	0.248	0.124	−0.059	-	-	-	-	-	-	-	-	-
aac(2′)	-	-	-	-	−0.273	−0.125	−0.083	0.035	-	-	-	-	-
aacA4	-	-	-	-	0.331	0.035	0.281	−0.053	-	-	-	-	-
aadA	-	-	-	-	0.427	0.353	0.105	−0.059	-	-	-	-	-
erm(B)	-	-	-	-	-	-	-	-	−0.047	-	-	-	-
tet(A)	-	-	-	-	-	-	-	-	-	−0.156	-	-	-
tet(B)	-	-	-	-	-	-	-	-	-	−0.347	-	-	-
sul1	-	-	-	-	-	-	-	-	-	-	-0.174	-	-
sul2	-	-	-	-	-	-	-	-	-	-	-0.343	-	-
qnrB	-	-	-	-	-	-	-	-	-	-	-	−0.106	0.035

AMP – ampicillin; AMX – amoxicillin; CRO – ceftriaxone; CFZ– cefazolin; GEN– gentamicin; STR – streptomycin; NER – neomycin; AMI – amikacin; EM – erythromycin; DOX – doxycycline; SXT – trimethoprim-sulfamethoxazole; CIP – ciprofloxacin; ENR – enrofloxacin
Pearson's correlation coefficients (r) shown in bold are significant at $P < 0.05$
A positive r indicates a positive association between the two variables, whereas a negative r indicates a negative association
- represents antimicrobials without corresponding antibiotic resistance genes

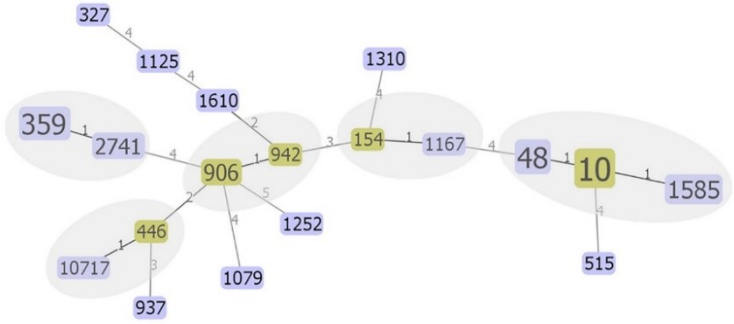

Fig. 1. Full minimum spanning tree using the goeBURST algorithm (n = 40). Each square represents a single sequence type (ST), and the circumference is proportional to the number of isolates within each ST. Grey regions represent a clonal complex. The numbers above the lines (1–5) represent the number of different alleles between the two ST types. The major nodes are indicated by in olive green

The associations of resistance genes and the corresponding antibiotics. The relationship between resistance genes and the corresponding antibiotics of *E. coli* strains was evaluated (Table 4). The results showed that four kinds of antimicrobials, namely amoxicillin (AMX), ceftriaxone (CRO), gentamicin (GEN), and streptomycin (STR), correlated positively with their corresponding resistance genes in *E. coli* strains ($P < 0.05$).

MLST and phylogenetic analyses. Nineteen STs were identified among all the 40 strains, of which the most prevalent was ST10 (n = 9, 22.5%), followed by ST48 (n = 6, 15.0%), ST359 (n = 5, 12.5%) and ST1585 (n = 3, 7.5%); 13 STs presented only once. No new STs were found in this study (Table 3). Five major nodes comprising 14 *E. coli* isolates were found by minimum spanning tree analysis of all ST types. Based on the single-locus variant level, 40 *E. coli* strains were classified as five clonal complexes (CC10, CC154, CC359, CC446 and CC906) and their main sequence types were ST10, ST154, ST359, ST446 and ST906, respectively. There was only one pair of allelic differences between neighbouring ST types in these clonal complexes (Fig. 1).

The evolutionary tree demonstrated a close genetic relationship between strains in the same clonal complex, such as CC154 (purple area, Fig. 2) and CC446 (yellow area, Fig. 2). The strains in CC10 (blue area, Fig. 2) were distributed in different clusters of the evolutionary tree, while ST48 strains were more distantly related to ST10 and ST1585 strains (Fig. 2). By antimicrobial resistance analysis, it was shown that there were differences in drug resistance profile and gene carriage in the same ST or CC strains. For example, two *E. coli* strains in CC446 (ID17 and ID19) (Fig. 2) had similar drug resistance profiles and carried a similar number of resistance genes, and the five ST359 strains (ID3, ID7, ID8, ID9 and ID12) (Fig. 2) also matched each other in the same manner. The reverse trend was found for two strains in CC154 (ID26 and ID28) (Fig. 2) and six ST10 strains (ID1, ID10, ID11, ID23, ID30 and ID34) (Fig. 2): compared with the other strains in the same CC group or the same ST type, these strains showed remarkable differences in their drug resistance profiles and numbers of antimicrobial resistance genes (Fig. 2).

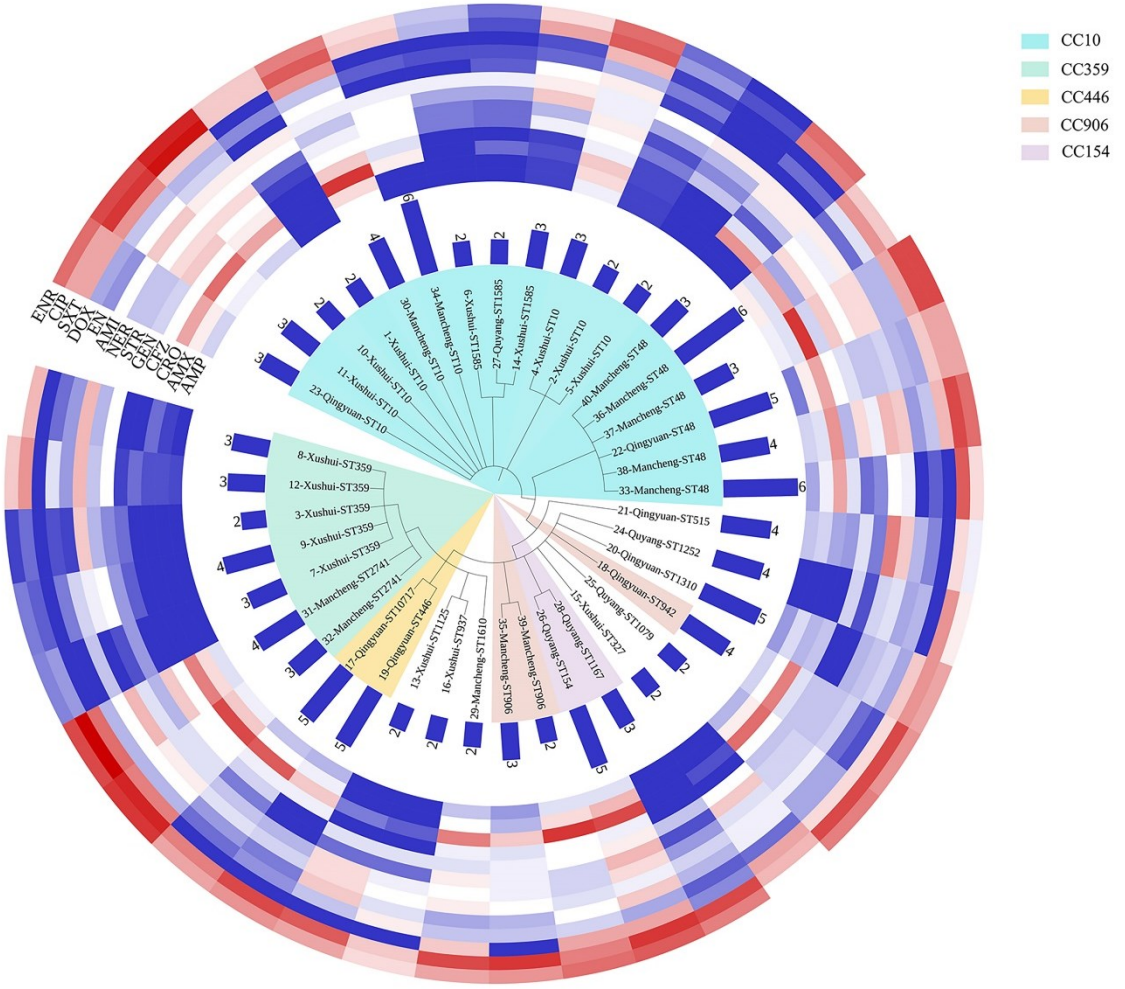

Fig. 2. Molecular phylogenetic and antimicrobial resistance analysis of 40 *E. coli* isolates. The evolutionary tree was inferred using the maximum likelihood method based on the Tamura–Nei model. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analysed. The branches of the evolutionary tree were named with ID, location, and ST type of *E. coli* isolates. The same clonal complexes were highlighted in the same colour area. The height of the blue bar graph on the periphery of the evolutionary tree represents the number of drug-resistant genes (2–6) carried by *E. coli* isolates. The diameter of different antibiotic inhibition zones (6–34 mm) was displayed as a heat map where red represents high-resistance diameters (trending to susceptible) and blue represents low-resistance diameters (trending to resistant)

Discussion

Escherichia coli is a primary environmental bacterium that can cause mastitis in dairy cow herds. A previous review indicated that dairy cows with mastitis caused by *E. coli* generally showed severe clinical signs such as redness, swelling, pain, and fever, and that even death could result from the disease (10). In response to coliform mastitis outbreaks or the threat of them in cattle herds, large amounts of antibiotics have been used worldwide. The overuse of antibiotics is severe in China. Statistically, more than 23% of antibiotics used in the world for food animal production were used in China, and the proportion is projected to increase to 30% in 2030 (5, 34). This questionable practice has led to the emergence of AMR in *E. coli* strains. Unfortunately, the adverse effects of antimicrobial resistance in bacteria have not attracted enough attention in livestock production (31). There are complicated genetic relationships among *E. coli* strains because of the clonal complex. Exploring these can provide helpful information to better understand the rules of antimicrobial resistance in a region. Therefore, it is constructive to analyse the perspective for *E. coli* antimicrobial resistance on dairy farms from the starting point of the phylogenetic community.

In this study, the isolation rate of *E. coli* in milk samples in the northern China region was 20.41%, which was higher than that of previous reports (7.8%) (6). Such a difference might be related to sample sizes, regional differences and detection methods. The results of susceptibility testing showed that the proportion of MDR strains was as high as 72.5%, which is higher than the 40% rate reported in milk samples from Egypt (31) but lower than the 87.8% reported in the Middle East (41). As was recently reported by Cheng *et al.* (9), MDR

E. coli in bovine mastitis is a problem in sizeable Chinese dairy herds. Most *E. coli* strains exhibited broad resistance to the beta-lactam antibiotics cefazolin (77.5%), ampicillin (52.5%), ceftriaxone (47.5%) and amoxicillin (45.0%), whereas the opposite situation was seen for neomycin (2.5%) and amikacin (2.5%) which are aminoglycoside antibiotics. In comparison, a study on the antimicrobial susceptibility of nine udder pathogens isolated from bovine clinical mastitis milk in Europe showed a higher resistance rate to ampicillin and tetracycline and a lower one to the beta-lactam antibiotics amoxicillin/clavulanic acid and cefazolin (12). We found apparent differences in antimicrobial consumption patterns between Europe and China (13), reflecting a combination of factors including pharmaceutical marketing strategies, veterinarian prescription patterns, governmental guidelines for proper antimicrobial use and farm economic benefit in different regions (33). Therefore, we inferred that our finding arose from the inappropriate use of antibiotics on the farms in the investigated region. Moreover, we found that resistance had emerged to gentamicin that was hitherto usually effective, which could be explained by the long-term use of this antibiotic in dairy herds (19).

There are many mechanisms of antimicrobial resistance in bacteria. In most cases, the presence of antimicrobial resistance genes strongly correlated with resistant phenotypes (3, 22), and a genetic origin aggravates the problem of antimicrobial resistance (21) because antimicrobial resistance genes can be transmitted both vertically and horizontally by plasmid, transposon and integrator in bacterial populations. This study found that more than half of the isolates carried the *aadA* and *tet*(B) genes and none carried the *blaTEM* and *erm*(C) genes, which is consistent with a previous study conducted in the north-eastern region of Jordan demonstrating the relatively high rate of *E. coli* of carriage of these resistance genes (17). To investigate the effects of difference in resistance gene distribution on antibiotic resistance in *E. coli* strains (8), we analysed correlations between antibiotics and corresponding resistance genes of *E. coli* isolates. The results showed that resistance to amoxicillin and ceftriaxone was correlated positively with resistance genes to beta-lactams (Table 4). This association is considered the main reason for the widespread resistance of *E. coli* to beta-lactam antibiotics on the regions' farms: although a large number of beta-lactam resistance genes were not detected, this trend could be the result of carriage of other genes of resistance to beta-lactams and also attributable to other complicated resistance mechanisms, such as extended-spectrum beta-lactamase production (4). Furthermore, some *E. coli* strains show no association between the antibiotics they resist and the corresponding resistance genes. Liu *et al.* (22) reported that most antibiotic resistance genes showed no correlations with their corresponding/non-corresponding antibiotics in conferring the expected resistance except the *tet*(A) resistance gene in bacteria from river drinking-water sources. Our results are only partially consistent with these findings. Perhaps environmental selection pressure affects the diversity and dissemination of antimicrobial resistance in *E. coli* strains (22, 27). In a further study, the relationship between the antimicrobial resistance of *E. coli* and various environmental factors needs to be clarified.

Multilocus sequence typing is a robust and reproducible method for analysing genetic relationships in population genetics and is frequently used in molecular epidemiological investigations (29). In this experiment, 40 *E. coli* strains were divided into 19 STs and 5 CCs. Most of the strains with the same ST type or CC had similar genetic relationships, which is in line with the with the previous study (43) and is evident in the minimum spanning tree and phylogenetic tree (Figs 1 and 2). Interestingly, unlike other CCs in the phylogenetic tree, ST48 strains have a distant relationship with other ST strains in CC10. However, there is only a pair of housekeeping genes' (*adk*) difference between ST48 and ST10 strains on the minimum spanning tree, and we speculate that this phenomenon is caused by the difference in the *adk* sequence (38). We also input the heat map of antimicrobial susceptibility and the number of resistance genes into the phylogenetic tree and found that the molecular characteristics were very different in the same CCs or in the same ST strains; this is consistent with reports that the antimicrobial sensitivity of *E. coli* differs greatly from region to region (16). We attribute the polymorphism of *E. coli* molecular characteristics to differences in the environment's hygiene level, farm management model and antibiotic use on dairy farms in different regions.

In conclusion, the present study elucidated the molecular characteristics of antimicrobial resistance and genetic correlations of *E. coli* from mastitic dairy cows in northern China. The farms in our study area were contaminated with MDR *E. coli*, which could have been caused by the inappropriate use of antibiotics. The high detection rates of MDR isolates and the differences in resistance suggested that measures should be taken to reduce the risk to animal food safety and human health, such as the use of only those antimicrobials which are prudent having regard to the AMR *E. coli* and genotypes on northern Chinese dairy farms.

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