An Epidemiological Study of Drug Resistance and Resistance Genes in Bovine *Escherichia coli* Isolates in Heilongjiang Province of China

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ABSTRACT

Background: To explore the epidemiology of bovine multidrug-resistant *Escherichia coli* isolates and resistance genes in Heilongjiang province of China. This study examined the prevalence of genes in bovine *E. coli* isolates, which confer resistance to antibiotics that are commonly used in the clinic, in regions of Baiquan, Shangzhi, and Songbei of Harbin. The purpose of the study was to investigate the epidemiology of the main resistance genes of bovine *E. coli* isolates in clinical veterinary medicine, and to provide a theoretical basis for preventing the spread of drug-resistant bacteria, as well as for rational drug use.

Materials, Methods & Results: The sensitivity of 105 isolates to 22 antibiotics was determined using the KirbyBauer disk diffusion method, and the distribution of 19 kinds of common drug resistance genes was investigated using Polymerase Chain Reaction. The results showed that the resistance rate to nine antibiotics was over 50%, including rifampin (84.76%), ampicillin (73.58%), tetracycline (69.52%), and sulfisoxazole (59.05%). In total, 105 strains of bovine *E. coli* presented 21 spectra of drug resistance, including eight strains (7.62%, 8/105) that were resistant to one antibiotic and four strains (3.81%, 4/105) that were resistant to 21 antibiotics. The resistance gene detection results showed that the streptomycin-resistance gene strA was found in 73 isolates, accounting for 69.52% of the isolates, followed by the sulfanilamide-resistance genes sul3/sul2 and the aminoglycoside-resistance gene aphA, which accounted for 57.14%, 51.43%, and 50.48%, respectively, of the isolates.

Discussion: This study revealed serious drug resistance of bovine *E. coli* isolates in some areas of Heilongjiang province. Of 105 *E. coli* isolates, more than 50% were resistant to the following antibacterial drugs: rifampicin, ampicillin, tetracycline, sulfisoxazole, and cephalothin. The isolates were the most sensitive to amikacin, with a sensitivity of 84.76%, followed by sensitivity to ofloxacin, ciprofloxacin, norfloxacin, cefoxitin, and tobramycin. Drug sensitivity tests showed that the drug resistance spectra of the bovine *E. coli* isolates was different in different regions, indicating that there were multidrug-resistant bovine *E. coli* isolates in different regions of Heilongjiang province, and that drug resistance differed among different regions. This may be due to prolonged use or overuse of antibiotics in a particular locality. Additionally, because of different management modes of livestock farms, the application of antimicrobial drugs in some farms may have imposed selective pressure on the intestinal flora including *E. coli*, resulting in the horizontal transmission of drug resistance among the bacteria. The study found that some strains had a resistance phenotype, but no resistance gene, while some had a resistance gene without expressing a resistance phenotype, which is consistent with relevant reports in the literature. This may be related to the same genotype corresponding to different resistance phenotypes, or different levels of gene expression, or different drug metabolic rates. In our study, some strains with certain drug resistance genes were sensitive to the corresponding drug, which may be due to mutations of drug-resistance genes, the loss of a strains resistance phenotype, or the loss of gene function. These issues require further study. This study revealed serious drug resistance of bovine *E. coli* isolates in some areas of Heilongjiang province. Of 105 *E. coli* isolates, more than 50% were resistant to the following antibacterial drugs: rifampicin, ampicillin, tetracycline, sulfisoxazole, and cephalothin. The isolates were the most sensitive to amikacin, with a sensitivity of 84.76%, followed by sensitivity to ofloxacin, ciprofloxacin, norfloxacin, cefoxitin, and tobramycin.

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INTRODUCTION

Escherichia coli is a pathogen that causes anthropozoonoses, which result in serious hazards to the livestock industry, food production, and environmental water sources [4]. *E. coli* is prone to drug resistance. Drug-resistant strains are gradually increasing in the clinic, and the drug resistance spectrum is expanding. Meanwhile, the residual presence of antimicrobial drugs in animals, as well as the spread of drug-resistant strains among different animals, adversely affects the sustainable development of the livestock industry, as well as public health.

The selective pressure of antibiotics can lead to drug-resistant *E. coli* in the gut of animals [20]. *In vivo*, *E. coli* not only obtains drug resistance through gene mutation and by capturing exogenous genes, but also participates in horizontal and vertical transmission of resistance genes, thus becoming a potential reservoir of drug-resistant genes in animals [18]. *E. coli* has been identified as an indicator strain for drug resistance tests, which is important for monitoring drug resistance [1]. Therefore, the detection of resistance genes is important to prevent bacterial resistance and to explore the mechanism of bacterial resistance.

This study examined the prevalence of genes in bovine *E. coli* isolates, which confer resistance to antibiotics that are commonly used in the clinic, in three regions Heilongjiang province of China (Baiquan, Shangzhi, and Songbei of Harbin). The purpose of the study was to investigate the epidemiology of the main resistance genes of bovine *E. coli* isolates in clinical veterinary medicine, and to provide a theoretical basis for preventing the spread of drug-resistant bacteria, as well as for rational drug use.

MATERIALS AND METHODS

Clinical isolates of Escherichia coli *strains*

The strains were isolated from rectal swabs and stools from calves with diarrhea and healthy cattle on 5 farms in three regions of Heilongjiang province of China (Baiquan, Shangzhi, and Songbei of Harbin). The samples were streak-inoculated onto MacConkey agar and eosin methylene blue agar and cultured at 37°C for 24 h. The resulting colonies were picked and identified as *E. coli* by biochemistry and Polymerase Chain Reaction. The quality control *E. coli* strain ATCC 25922 was purchased from the China Industrial Microorganism Culture Collection Center.

Test drugs

Amoxicillin (AMC), ampicillin (AMP), cefoxitin (CFX), ceftriaxone (CRO), ceftazidime (CAZ), cephalo-thin (CF), streptomycin (S), kanamycin (K), gentamicin (GM), tobramycin (TM), amikacin (AMK), sulfamethoxazole (SXT), sulfisoxazole (SIZ), chloramphenicol (C), florfenicol (FFC), ciprofloxacin (CIP), norfloxacin (NOR), ofloxacin (OFL), nalidixic acid (NAL), tetracycline (TC), doxycycline (DOX), and rifampin (RA)[1].

Drug sensitivity tests

Drug sensitivity tests were performed with the Kirby-Bauer disk diffusion method using Mueller-Hinton agar. Bacteria were inoculated into BHI[2] medium and shaking-cultured at 37°C for 10 h. Then, normal saline was added to adjust the cultures to 0.5 McFarland units, the same as that of the standard tube, for comparative tests. Within 15 min, a sterile cotton swab was dipped into the culture broth and spread on a Mueller-Hinton agar plate[2]. Under aerobic conditions, the plate was cultured at 37°C for 24 h. *E. coli* ATCC 25922 served as a quality control strain, the diameter of the bacteriostatic zone was measured by a Vernier caliper, and the sensitivity of the isolates to antibacterial drugs was assessed according to American Committee for Clinical Laboratory Standards.

Resistance gene testing of isolates

Based on the drug resistance mechanisms of *E. coli* against β-lactams, aminoglycosides, tetracyclines, streptomycin, sulfonamides, quinolones, and chloramphenicol, 19 resistance genes, including SHV genes, floR, and aacA4, were selected to analyze the genotypes of the resistance genes from the bovine *E. coli* isolates. Amplification products were processed via 1.0% agarose gel electrophoresis (1 × TAE[3], with ethidium bromide (EB)[3] staining, for 20 min at 120 V) and photographed with a gel-documenting system[4]. PCR primers and annealing temperatures are shown in Table 1.

RESULTS

Drug sensitivity of bovine Escherichia coli *isolates*

A total of 105 bovine E. coli strains showed varying degrees of resistance to 22 antibiotics, among which the resistance rates to 7 antibacterial drugs were greater than 50%. The resistance rate to RA was the highest, 84.76%, followed by AMP (73.58%), TC (69.52%), and SIZ (59.05%). The sensitivity rates of the strains to 14 antibacterial drugs were more than 50%, ranked as follows: AMK (84.76%), OFL (80.00%), CIP (76.92%), NOR (76.19%), and FFC (75.24%). The details are listed in Table 2.

Table 1. Primers used in Resistance gene detection of *Escherichia coli* isolated from cattle.

Antimicrobial family and gene	Sequence (5'-3')	Amplicon size (bp)	Annealing T	GenBank accession no.
bla-CMY-2	GACAGCCTCTTTCTCCACA TGGAACGAAGGCTACGTA	1 014	56	NG_041242.1
bla-TEM	ATGAGTATTCAACATTTCCG CTGACAGTACCAATGCTTA	860	58	AF309824
bla-SHV	TCGCCTGTGTATTATCTCCC CGCAGATAAATCACCACAATG	873	56	AF148850
bla-CTX	CGATGTGCAGTACCAGTA A TTAGTGACAGAATCAGCGG	566	57	JN411912.1
strA	CCTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	539	56	EF090911.1
strB	ATCGTCAGGGATTGAAACC GGATCGTAGAACATATTGGC	489	57	EF090911.1
tetA	CGGCAGGCAGAGCAAGTAGA GGCGGTCTTCTTCATCATGC	482	58	X54723
tetB	CATTAATAGGCGCATCGCTG TGAAGGTATCGATAGCAGG	514	56	EF646764.1
Sul1	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	770	58	M62822
Sul2	CGGCATCGTCAACATAACCT TGTGCGGATGAAGTCAGCTC	720	58	X53796
Sul3	ACCTAAGAATGATTTCCGTGAC ACTGAAGTGGGCGTTGTG	313	57	AY494779.1
floR	ACGTTTATGCCAACCGTCCTG AAAGTGCCACCGCCAATGTCC	495	58	X00006
clmA	CGCCCGGACTAATGAT GCAATAAAGCCTAACACG	229	56	AB212941.1
Apha1	CATGACCTTGCGATGCTCT CTGGTCTATTCCGCGTACTCC	258	55	X92506
aadB	TGTAACACGCAAGCACGATG AACCTTTTCCGCCCCGAGT	285	58	NG_035375.1
aacA4	CATGACCTTGCGATGCTCT CTGGTCTATTCCGCGTACTCC	635	56	KC417377.1
qnrA	TCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCA	627	55	KC493118.1
qnrS	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	428	58	NG_039601.1
qepA	GCAGGTCCAGCAGCGGGTAG CTTCCTGCCCGAGTATCGTG	199	55	FJ167861.1

Table 2. The results of drug sensitivity test of 105 *Escherichia coli* isolated from cattle.

Antimicrobial		Resistant	Intermediate	Sensitive	Sensitive rate	Intermediary rate	Resistance rate
β -lactams	AMC	37	10	58	55.24%	9.52%	35.24%
	AMP	78	4	23	21.90%	3.81%	74.29%
	CFX	18	8	79	75.24%	7.62%	17.14%
	CRO	50	8	47	44.76%	7.62%	47.62%
	CAZ	20	10	75	71.43%	9.52%	19.05%
	CF	57	12	36	34.29%	11.43%	54.29%
Aminoglycosides	S	51	13	41	39.05%	12.38%	48.57%
	K	43	7	55	52.38%	6.67%	40.95%
	GM	30	3	72	68.57%	2.86%	28.57%
	TM	22	7	76	72.38%	6.67%	20.95%
	AMK	7	4	94	89.52%	3.81%	6.67%
Sulfonamides	SXT	44	2	59	56.19%	1.90%	41.90%
	SIZ	62	0	43	40.95%	0.00%	59.05%
Chloramphenicols	C	41	8	56	53.33%	7.62%	39.05%
	FFC	24	2	79	75.24%	1.90%	22.86%
Quinolones	CIP	22	1	80	77.67%	0.97%	21.36%
	NOR	22	3	80	76.19%	2.86%	20.95%
	OFL	19	2	84	80.00%	1.90%	18.10%
	NAL	41	3	61	58.10%	2.86%	39.05%
Tetracyclines	TC	63	3	39	37.14%	2.86%	60.00%
	DOX	40	3	62	59.05%	2.86%	38.10%
Rifamycin	RA	89	10	6	5.71%	9.52%	84.76%

Amoxicillin (AMC), ampicillin (AMP), cefoxitin (CFX), ceftriaxone (CRO), ceftazidime (CAZ), cephalothin (CF), streptomycin (S), kanamycin (K), gentamicin (GM), tobramycin (TM), amikacin (AMK), sulfamethoxazole (SXT), sulfisoxazole (SIZ), chloramphenicol (C), florfenicol (FFC), ciprofloxacin (CIP), norfloxacin (NOR), ofloxacin (OFL), nalidixic acid (NAL), tetracycline (TC), doxycycline (DOX), and rifampin (RA).

Drug resistance spectra of bovine Escherichia coli *isolates in different regions*

In three areas of Heilongjiang province, a total of 105 bovine *E. coli* strains showed 21 drug resistance spectra, in which 8 strains (7.62%, 8/105) were resistant to 1 drug, and 4 strains (3.81%, 4/105) were resistant to 21 drugs. In the Shangzhi area, strains resistant to 13 or 15 drugs accounted for 15.91% of the strains, while strains resistant to 17, 19, or 1 drug accounted for 11.36% of the strains. In the Baiquan area, 34.88% of the strains were resistant to one drug, followed by strains resistant to 5, 3, or 13 drugs, accounting for 25.58%, 23.26%, and 9.30% of the strains, respectively. In the Songbei area, strains resistant to 7 or 11 drugs accounted for 22.22% of the strains, followed by strains resistant to 9 (16.67%), 15 (11.11%), and 19 drugs (11.11%) [Figure 1].

Resistance phenotypes of bovine Escherichia coli

Of the 105 bovine *E. coli* isolates, there were mainly 15 resistance phenotypes to nine antibiotics.

AMP + S + TC + C + SIZ and AMP + SIZ resistant phenotypes accounted for 10.48% of the isolates (the maximal proportion), followed by resistance to AMP + CF + SIZ + S + TC, AMP + CF + SIZ + TC + S + GM, or AMP + CF + TC + S + GM + C, accounting for 8.57% of the total isolates (Table 3).

Resistance genes of bovine Escherichia coli *isolates*

Of the 105 bovine *E. coli* isolates, the test results for 19 resistance genes in 7 categories showed that the sulfonamide-resistant gene sul3 was the most widely distributed, as it was found in 30 isolates (28.57%), followed by the chloramphenicol-resistant gene floR, which was found in 28 strains (26.67%), the β-lactam-resistant gene blaTEM (25 strains, 23.18%), and the streptomycin-resistant gene strA (20 strains, 19.05%). Five strains carried the quinolone-resistant gene qepA, accounting for 4.76% of the strains (the lowest frequency), followed by the sulfonamide-resistant gene sul1 (6.67%) and the chloramphenicol-resistant gene clmA (7.62%) [Table 4].
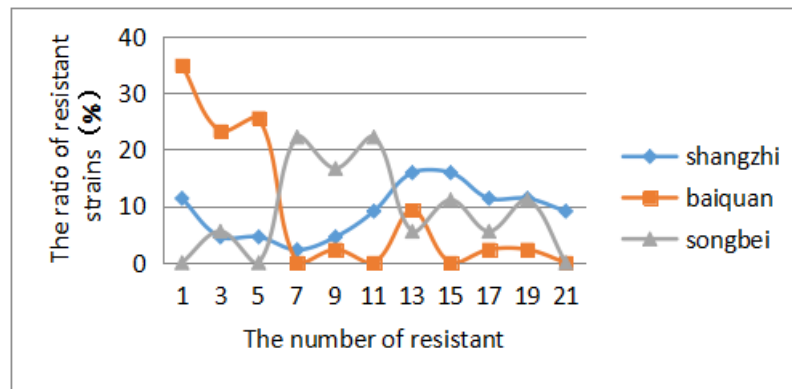
Figure 1. Drug resistance spectrum of *Escherichia coli* from cattle in different areas of Heilongjiang province of China

Table 3. Nine kinds of antimicrobial drug resistance phenotype of 105 *Escherichia coli* isolated from cattle.

Phenotypic resistance	Isolates	Percentage
AMP+CF	8	7.62%
AMP+SIZ	11	10.48%
AMP+CF+S+SIZ	7	6.67%
AMP+CF+SIZ+S+TC	9	8.57%
AMP+SIZ+S+TC+GM	7	6.67%
AMP+S+TC+C+SIZ	11	10.48%
AMP+CF+SIZ+TC+S+GM	9	8.57%
AMP+CF+TC+S+GM+C	9	8.57%
AMP+CF+TC+S+GM+C+SIZ+CIP	5	4.76%
AMP+TC+S+C+SIZ+CIP	4	3.81%
CF+TC+GM	2	1.90%
CF+S+TC+C	5	4.76%
CF+S+TC+C+SIZ	8	7.62%
CF+S+TC+C+GM +SIZ	8	7.62%
AMP+CF+S+C+GM+TC+SIZ+CIP+AMK	2	1.90%

Ampicillin (AMP); cephalothin (CF); sulfisoxazole (SIZ); streptomycin (S); tetracycline (TC); gentamicin (GM); chloramphenicol (C); streptomycin (S); ciprofloxacin (CIP).

Table 4. Carrier rate of different resistant genes of 105 *Escherichia coli* isolated from cattle.

Resistance gene	Isolates	Carrier rate
blaCTX	0	0.00%
blaCMY	16	15.24%
blaTME	25	23.81%
blaSHV	9	8.57%
aacA4	19	18.10%
aphA	11	10.48%
aadB	16	15.24%
sul1	7	6.67%
sul2	15	14.29%
sul3	30	28.57%
tetA	17	16.19%
tetB	11	10.48%
floR	28	26.67%
clmA	8	7.62%
qnrs	16	15.24%
qnrA	9	8.57%
qepA	5	4.76%
strA	20	19.05%
strB	17	16.19%

Resistance genotypes of bovine Escherichia coli *isolates from different areas*

Of the 105 bovine *E. coli* isolates from the 3 areas, there were 7 kinds of resistant genotypes, including resistance to β-lactams, aminoglycosides, sulfonamides, tetracycline, chloramphenicol, quinolones, and streptomycin. Despite the presence of the same genotypes in all three areas, there were some differences between the different areas regarding the number of isolates carrying drug resistance genes (Table 5).

Correlation between resistance phenotype and resistance genes

Consistency analysis of the resistance phenotypes and the resistance genes to nine antibiotics showed that sulfamethoxazole had the highest consistency (83.87%), followed by chloramphenicol (68.29%), while norfloxacin had the lowest consistency (22.72%). It was observed that some isolates presented drug resistance without carrying resistance genes, whereas some isolates carried resistance genes without manifesting a resistance phenotype (Table 6).

Table 5. The distribution of resistant genes of *Escherichia coli* isolated from cattle fom regions of Baiquan, Shangzhi, and Songbei of Harbin - Heilongjiang province of China.

GeneType	β -lactams				Aminoglycosides			Sulfonamides			Tetracyclines		Chloramphenicols		Quinolones			Streptomycin	
	CTX	CMY	TME	SHV	aacA4	aphA	aadB	sul	sul2	sul3	tetA	tetB	floR	clmA	qnrs	qnrA	qepA	strA	strB
shangzhi	0	13	12	6	10	9	6	4	7	15	13	0	19	7	14	5	2	12	11
baiquan	0	3	8	3	5	2	6	3	6	7	2	4	3	1	2	1	0	3	1
songbei	0	0	5	0	4	0	4	0	2	8	2	7	6	0	0	3	3	5	5

Table 6. Comparison of consistency in *Escherichia coli* isolates according to phenotypic and genotypic testing.

Antimicrobial	Characteristics of isolate					
	N_P^*	Gene(s) carried	N_G^*	consistency	P+/G-*	P-/G+*
Ampicillin	78	blaTEM blaSHV	25 9 34	43.59%	58	2 3
Cefalotin	57	blaCMY-2	21 21	36.84%	29	0
Sulfamethoxazole	62	sul1 sul2 sul3	7 15 30 52	83.87%	8	0 3 9
Kanamycin	43	aphA aadB	11 16 27	62.80%	30	3 7
chloramphenicol	41	floR	28 28	68.29%	10	1 2
norfloxacin	22	qepA	5 5	22.72%	8	1
tetracycline	63	tetA tetB	17 11 28	44.44%	23	2
streptomycin	51	strA strB	20 17 37	72.55%	17	5

N_P^*: number of *E. coli* isolates expressing phenotypic resistance to the indicated antimicrobial agent; N_G^*: number of *E. coli* isolates carrying the indicated resistance gene; P+/G-*: number of phenotypically resistant *E. coli* isolates (P+) with no resistance gene (G-) for the drug identified; P-/G+*: number of phenotypically susceptible *E. coli* isolates (P-) with a resistance gene (G+) for the drug identified.

DISCUSSION

The drug resistance of *Escherichia coli* is mainly acquired via mutation or the capture of exogenous resistance genes by plasmids, transposons, and other removable fragments, and exogenous genes play a major role in bacterial resistance [19].

β-lactamase plays a key role in the drug resistance of E. coli to broad-spectrum β-lactams [3,12]. This study examined four kinds of β-lactam-resistance genes and found that more isolates carried blaTEM and blaCMY genes, while fewer isolates carried blaCTX and blaSHV genes. Of the 105 bovine *E. coli* isolates in our study, the overall detection rate of blaTEM was 15.24% (16/105), which was higher than that reported in China a few years ago [6,23], indicating that due to the increased use of cephalosporins, there was a rising trend of extended spectrum β lactamases (ESBLs) produced by bovine *E. coli* isolates.

The aminoglycoside-resistance gene aacA4 had the highest detection rate, and the resistance genes aacA4, aphA, and aadB were detected in all three regions, which was different from a previous report. This suggests that there are some differences in the distribution of aminoglycoside-resistance genes in different regions.

The streptomycin-resistance genes strA and strB co-mediate the streptomycin-resistance phenotype [17]. In this study, the detection rates of strA and strB were 19.05% and 16.19%, respectively, indicating that almost all strains carrying strA also carried strB; thus, the consistency of the results was good.

It was reported that the most common tetracycline-resistance phenotype had more than 40 kinds of coding genes, but the two efflux pump genes, tetA and tetB, accounted for 90% of the resistance [16], which was also reported in gut commensal *E. coli* and pathogenic *E. coli* [10,11]. In this study, the detection rates of tetA and tetB were 16.19% and 10.48%, respectively. However, only tetA was detected when testing tetracycline-resistance genes in the Sangzhi area, probably because the incompatibility between plasmids resulted in a negative correlation between tetB and tetA [2].

The test results for the chloramphenicol resistant genes floR and clmA showed that although chloramphenicol use is forbidden in livestock animals in China, the survey found a high degree of resistance to chloramphenicol, with a positive rate of 34.29%. We suspected that the genes encoding chloramphenicol resistance were co-screened and preserved under selective pressure related to other antimicrobial agents. One study demonstrated that strains carrying floR also showed resistance to chloramphenicol, while strains carrying cmlA were not 100% resistant to florfenicol [22], which was consistent with the results of our study. Therefore, the mechanism of chloramphenicol resistance still needs to be further explored.

The results of sulfonamide-resistance gene tests showed that the detection rates of sul1, sul2, and sul3 were 6.67%, 14.29%, and 28.57%, respectively. In 2003, sul3 was first discovered by Perreten *et al*. [13]. Afterwards, sul3 was found in *E. coli* strains in many countries. It was reported [7,8] that the main sulfonamide-resistance genes in bovine *E. coli* isolates were sul1 and sul2; the detection rate of sul2 was two to five times that of sul1, and only a few sul3 genes were observed. This was quite different from our study, in which the detection rate of sul3 was the highest, and the detection rate of sul2 was two times higher than that of sul1.

Since 1998, three plasmid-mediated quinolone resistance determinants including qnr genes (qnrA, qnrB, qnrS), aac(6')-Ib-cr, and qepA, have been reported in various Enterobacteriaceae genera worldwide [9]. qnr encodes the QNR protein, which can protect DNA gyrase and topoisomerase IV from inhibition by quinolones; a qepA-encoded plasmid-mediated efflux pump can mediate low-level drug resistance by excreting hydrophilic quinolones [15]. In this study, the detection rates of qnrS, qnrA, and qepA in 105 bovine *E. coli* isolates were 15.24%, 8.57%, and 4.76%, respectively, indicating that PMQR gene is prevalent in Heilongjiang province, and that qnrS is the dominant type. This is different from reports in recent years [14], which may be due to the different sources of strains and different medications. Studies have shown that qnr plasmids often carry integrons, transposons, and multi-drug resistance gene determinants, especially associated with ESBL-producing genes, which are helpful for the horizontal or vertical transmission of resistance genes among bacteria in the same or different genus under the selective pressure of different drugs [5]. Therefore, how to promote the scientific and rational use of quinolones to reduce the production of quinolone-resistant strains becomes an important issue in future research.

CONCLUSIONS

The results showed that drug-resistant bovine E. coli isolates were common in Heilongjiang province. Additionally, these isolates possessed diverse and complex drug resistance phenotypes, and drug resistance genes were widely distributed in drug-resistant strains; however, no significant association was observed between the drug resistance phenotype and resistance genes.

MANUFACTURERS

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