Original Article

Risk factors of bovine clinical mastitis and antimicrobial resistance of mastitis-causing *Escherichia coli* in Shandong, China

Peng Liu[1*] Dongfang Hu[2] Jinjiao Zuo[3] Caihong Wu[1]

Hui Lu[1] Zilong Cheng[4] Mengda Liu[5*]

Abstract

The culling of dairy cattle is closely related to the economic benefits of the farm. Many reasons can lead to the culling of cows. Among these, mastitis is the main reason for the serious economic loss of the dairy industry, and also the harm to global public health. However, little is known about cow culling situations, antimicrobial resistance, and virulence factors of mastitis-causing Escherichia coli in Shandong, China. A cross-sectional study was conducted in 16 randomly selected dairy herds to identify the reasons for cow culling, to assess the risk factors, and to isolate the major etiological agent of clinical mastitis (CM). The overall culling rate of dairy cows was 25.24%. And 81.66% of the culled cows occurred before their fourth parity. The most common reasons for culling were mastitis (32.91%) and reproductive failure (28.38%). The investigated herds had CM rates ranging from 4.17 to 10.64%. The filler material in cow beds, management of milking parlors, cleaning of quarters before milking, disinfection of teat cups every time, hygiene of breast and leg, and the nipple score were identified as risk factors associated with CM. From the 101 milk samples analyzed by cultured, 92 bacteria were yielded. The predominant bacteria were *Escherichia coli* (31.52%), *Streptococcus* species (27.17%), and *Staphylococcus aureus* (15.22%). The majority of the *E.coli* isolates, which belonged to phylogeny group A, were defined as commensal strains. Virulence genes iucD was detected in 91.67% of the *E.coli* isolated, followed by iss (83.33%). All of the 29 E.coli isolates were resistant to at least one antimicrobial tested. Resistance to ciprofloxacin or levofloxacin was most common (100%), which might be due to mutations (Ser83Leu/Asp87Asn) in the gyrA gene. Prevalence of multi-drug resistance (resistance to >3 antimicrobials) was 62.07% (18/29). This study emphasizes the need for prevention and control of mastitis to lower the culling hazard in Shandong. Moreover, it is necessary to strengthen the rational use of antimicrobials and infection control measures in dairy farms in order to reduce the emergence of drug-resistant bacteria.

Keywords: antimicrobial resistance, bovine mastitis, China, cow culling, risk factors

[1]*College of Veterinary Medicine, Jiangsu Agri-Animal Husbandry Vocational College, Jiangsu, 225300, PR China*
[2]*College of Animal Science and Technology, Henan Institute of Science and Technology, Xinxiang 453003, China*
[3]*Pet of Veterinary Medicine, Jiangsu Agri-Animal Husbandry Vocational College, Jiangsu, 225300, PR China*
[4]*Jiangsu Academy of Agricultural Sciences, Nan'jing, 210014, China*
[5]*Laboratory of Zoonoses, China Animal Health and Epidemiology Center, Qingdao 266032, China*
***Correspondence**: 2022010532@jsahvc.edu.cn* (P. Liu, M. Liu)
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Introduction

With the steady development of the dairy industry in China, more and more attention has been paid to dairy farming. The industrialization degree of the dairy industry in Shandong Province ranks at the forefront in China. However, there are still some problems in the development of the dairy industry in Shandong Province, such as low production levels and small scale. The culling of dairy cattle is closely related to the economic benefits of the farm, which is one of the most important issues for dairy farmers. Culling refers to the identification and removal of a cow from a cow farm. Assuming that the size of the herd is constant or expanding, another cow, usually a first-lactation heifer, is used to replace the cow. The culling rate describes the percentage of cows removed from the herd. Understanding the culling rate is important for managing the reaction and profitability of dairy production. When dairy managers cull cattle too often or too quickly, the cost of substitution is too high. While, milk production, reproduction, or genetic modification may be affected when the manager keeps the cattle for a long time(Hadley *et al*., 2006). Traditionally, an examination of culling patterns has included "voluntary" and "involuntary" culls. According to previous reports, cows were usually culled due to low production, illness, injury, infertility, or death (Adamczyk *et al*., 2017). Among them, reproduction and mastitis were found to be the most common reasons(Bell *et al*., 2010; Keane, 2016).

Mastitis refers to the inflammation of the mammary gland, resulting in a great deal of loss or reduction of milk yield to influence the development of the dairy industry(Sarba and Tola, 2017). Mastitis not only affects the lactation function and reproductive function of dairy cows but also results in the decrease of milk yield and quality, and endangers human health. Mastitis can be classified as clinical mastitis (CM) and subclinical mastitis. CM is characterized by changes in the morphology of the udder, chemical and physical changes in the milk. While the subclinical form, known as recessive mastitis, is without any noticeable manifestations of inflammation. Subclinical mastitis is more common than the CM and causes the greatest overall losses in most dairy herds. It's reported that the occurrence of mastitis in China can reach 20-32% in large-scale dairy farms, causing huge economic losses to the dairy industry(Dan *et al*., 2019). The health of mammary gland of dairy cow is affected by many factors, including environment, management, etiology, age, and parity. Among the factors, the most important one is pathogen infection, including pathogenic fungi, bacteria, mycoplasma, and virus. The majority of mammary infections are caused by bacteria namely *Escherichia coli (E.coli)* (Kaipainen *et al*., 2002; Suojala *et al*., 2011), streptococci and staphylococci.

E. coli has traditionally been considered as an opportunistic pathogen that can enter the breast through the nipple tube and cause infection (Keane, 2016). Four major phylogenetic groups including A, B1, B2 and D have been characterized and researchers found that distinct clonal groups of E.coli have been associated with a variety of human and animal diseases(Clermont *et al*., 2000; Ewers *et al*., 2009; Haine *et al*., 2017). Several virulence factors including toxins, adhesions, invasins, capsule production and the ability to resist serum complement that detected from pathogenic E.coli cause urinary tract infections, diarrhea, and septicemia in humans and animals (Kaipainen *et al*., 2002). For the purpose of disease prevention, antimicrobials are widely used in many dairy farms, which leads to the increase of drug residues in milk and the emergence of new drug-resistant strains, which brings many difficulties to the prevention and control of cow diseases(Zehra *et al*., 2017). Studies of antimicrobial resistance and virulence traits in *E.coli*, Streptococci and Staphylococci have been performed in milk of cows with mastitis in several countries. However, the risk factors, bacterial infection and drug resistance of cow mastitis are rarely reported. The incidence of antimicrobial resistance of bacteria isolated from raw milk in dairy herds in Shandong province is very limited.

At present, modern dairy farming is growing in cities and suburbs of major cities and towns in Shandong Province. In addition, milk production is expected to increase more rapidly due to the rapid growth of demand for milk and dairy products due to the increase of population in urban areas and the increase of consumer income. Therefore, it is necessary to master the epidemiological data and the factors related to mastitis, especially to characterize the mastitis-causing bacteria by antimicrobial susceptibility and key virulence genes, so as to improve the production of dairy products and maintain the milk quality for consumers.

Materials and Methods

Ethical approval: This study was approved by the Ethics Committee of Animal Experiments Center of Jiangsu Agri-Animal Husbandry Vocational College. All animal studies were approved by the Institutional Animal Care and Use Committee of Jiangsu Agri-Animal Husbandry Vocational College (jsahvc-2023-11), and followed the National Institutes of Health guidelines for the performance of animal experiments. In addition, permission was obtained from the owner to conduct the study.

Data collection and sampling: The study was conducted in 16 non-repetitively selected dairy herds in Shandong province. We first investigated the cow culling rates and the associated causes that were diagnosed by the attending veterinarian in each herd. Additionally, the parities of the culled cows were also recorded. A total of 1309 cows in the 16 herds were examined clinically by the field veterinarians, and the cows showed that CM was recorded and scored according to a 3-point scale as described earlier (Suojala *et al*., 2010). A cow with visible signs of mastitis, either, mild (flakes or clots in milk, slight swelling of infected quarter) or severe (abnormal secretion, hot, swollen quarter or udder, fever, rapid pulse, loss of appetite, dehydration and depression) signs was defined as CM.

Prior to the study, a questionnaire, which was used to identify the risk factors associated with CM, was designed according to experts' opinions and modified

by the attending veterinarians in dairy herds. The following items were assumed to be risk factors associated with bovine CM: herd size, management system, filler material in cow bed, type of feeding, management of milking parlors, the utilization rate of cow bed, cleaning of quarters before milking, drying quarters, disinfection of teat cups every time, lactation stage, milk yield, hygiene of breast and leg, and nipple score. The survey was conducted among field veterinarians and had a 100% response rate.

The milk sample was collected aseptically from the affected quarter of cows with CM according to a previous report (Kateete *et al*., 2013). The milk samples were stored briefly in an ice-cold box and promptly transported to the bacteriology laboratory for culture. Cows were clinically re-examined by field veterinarians to confirm symptoms prior to sample collection.

Bacterial isolation and identification of CM: Ten µL of milk was cultured on blood agar or on tryptic soy agar (for samples with no growth on blood agar plates) and the isolates were identified by the colony morphology and Gram staining (Hogan *et al*., 1999). Pure cultures were obtained and the bacterial pellet was re-suspended in 200 µL of distilled water and boiled for 10 min. Tubes were centrifuged again, and the supernatant was used as template DNA. A multiplex PCR was performed on the samples to identify the associated pathogens including Staphylococcus aureus, Streptococcus and E.coli. The primer sets and PCR condition for amplification are designed according to a previous report (Salmon *et al*., 2009).

Phylogeny and virulence genes detection of E.coli: The E.coli isolates were assigned to a phylogeny group by a simple and rapid phylogenetic grouping technique based on triplex PCR, which uses a combination of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (Clermont *et al*., 2000). The virulence genes to be examined were selected based on those of extra-intestinal pathogenic *E. coli* and entero-hemorragic *E. coli* (Suojala *et al*., 2011). The examined virulence genes including colisin V plasmid (*cva*), vacuolating autotransporter protein (*vat*), temperature-sensitive hemagglutinin (*tsh*), aerobactin (*iucD*), P-fimbria (*papC*), yersinia bactin (*irp2*), increased serum survival (*iss*), enteroaggregative heat-stable toxin (*astA*), shigatoxin 1 and 2 (*stx1* and *stx2*), intimin (*eaeA*), enterohemolysin (*ehxA*), autoagglutinating adhesin (*saa*), S-fimbria (*sfaD*), F17 fimbria (*f17A*), afimbrial adhesin AFA8 (*afa8E*) and cytotoxic necrotizing factor 2 (*cnf2*), and the genes were detected by corresponding PCR methods that described by previous reports (Paton and Paton, 1998; Kaipainen *et al*., 2002; Paton and Paton, 2002; Van Bost *et al*., 2003; Ewers *et al*., 2005).

Antimicrobial resistance analysis: Antimicrobial susceptibility testing was performed for the isolated E.coli by the disk diffusion method in accordance with Clinical and Laboratory Standards Institute guidelines (Watts, 2013). The E.coli ATCC 25922 strains were used for quality control standards, respectively. Isolates were classified as resistant, intermediate or susceptible based on a comparison of the measured zone diameter with the zone diameter interpretive standards for veterinary pathogens.

PCR detection of antibiotic resistance genes: The carriage of antibiotic resistance genes in E.coli isolates was analyzed using PCR assays. Carriage of extended-spectrum β-lactamase genes including $bla_{\text{CTX-M}}$, bla_{TEM} and $bla_{\text{SHV-1}}$ (De Gheldre *et al*., 2003; Haeggman *et al*., 2004; Birkett *et al*., 2007), macrolide resistance genes including *erm B*, *erm C* and *erm F* (Choo *et al*., 2018) was assessed by single or multiplex PCR. Moreover, PCR amplification of quinolone resistance-determining regions of the genes coding for gyrase (gyrA and gyrB) and topoisomerase IV (parE and parC) (Giraud *et al*., 1999) was performed on ten ciprofloxacin-resistant E.coli isolates. The PCR products were sequenced by Sangon Biotech (Shanghai, China). Sequence data were then analyzed by DNAstar (DNAstar Inc., Madison, WI, USA) and the sequences were compared with reference sequences from NCBI GenBank to find the occurrence of mutations.

Statistical analysis: Data are shown as the mean ± S.D. Data were analyzed using one-way ANOVA followed by LSD multiple comparison tests by SPSS 20.0 software (SPSS, Chicago, IL). A P value of less than 0.05 was considered statistically significant.

Results

Cow culling situations: The average cow culling rate for the 16 herds was 25.24 percent per year. The investigated herds had culling rates ranging from 8.11 to 39.38%. The average cow culling rate increased with herd size. Farms with a herd size of less than 500 cows had an average annual culling rate of 21.43%. The largest size group, herds with more than 1,000 cows, had the highest average annual culling rate of 31.63% (Fig.1A). Of the cows culled, 81.66% (975/1194) occurred before their fourth parity, and cows of the second parity had the highest culling rate of 37.60% (449/1194) (Fig.1B).

Examining culling reason revealed that the primary reason for cows being culled in Shandong province was mastitis (32.91%), with reproductive failure (28.38%) being the second most common reason, followed by feet and legs diseases (19.79%) (Fig.1C). The investigated herds had mastitis rates ranging from 4.17 to 10.64% and the average mastitis rate for the 16 herds was 7.6%. There were no significant differences of average mastitis rate among farms of different herd sizes (Fig.1D).

Risk factors associated with CM: As shown in Table 1, two herd factors (herd size and the filler material in cow bed), three management factors (management of milking parlors, cleaning of quarters before milking, and disinfection of teat cups every time), and two cow factors (hygiene of breast and leg and the nipple score) were identified as statistically significant risk factors of CM. This study revealed there was no significant difference in the farms with more than 1,000 cows as compared to smaller farms ($P > 0.05$). There was also a significant association ($P < 0.05$) between the filler material in cow beds and mastitis evidenced by lower

prevalence in herds that used sandy soil as the filler material in cow beds than in herds that used other materials (Table 1). Analysis of management factors showed lower mastitis rates ($P < 0.05$) in cows from the herds with excellent management of milking parlors, using disposable wipes to clean the quarters before milking and disinfecting the teat cups every time before using. As for the cow factors, the contamination of the breast and leg and the injury of the nipples were found to be significantly associated with the occurrence of CM ($P < 0.05$).

Bacterial distribution in milk samples: During this study, 101 bovine milk samples from cows with CM in Shandong province were studied for the distribution of bacterial species. One sample per animal was collected, representing a total of 101 cows. Bacteria grew from 77 milk samples (76.24%, 77/101) of which 64 (83.12%, 64/77) grew pure cultures. A total of 92 bacterial isolates were purified from the 77 milk samples and were submitted for further examination. There were 53 isolates of Gram-positive bacteria (53/92, 57.61%), of which 25 were Streptococcus (25/53, 47.17%) while 14 were Staphylococcus aureus (14/53, 26.42%). Thirty-nine (39/92, 42.39%) purified cultures were identified as Gram-negative bacteria and twenty-nine of them were identified as E.coli (29/39, 74.36%). These results indicated that E.coli (29/92, 31.52%) and Streptococcus (25/92, 27.17%) are the predominant bacteria associated with CM in Shandong province.

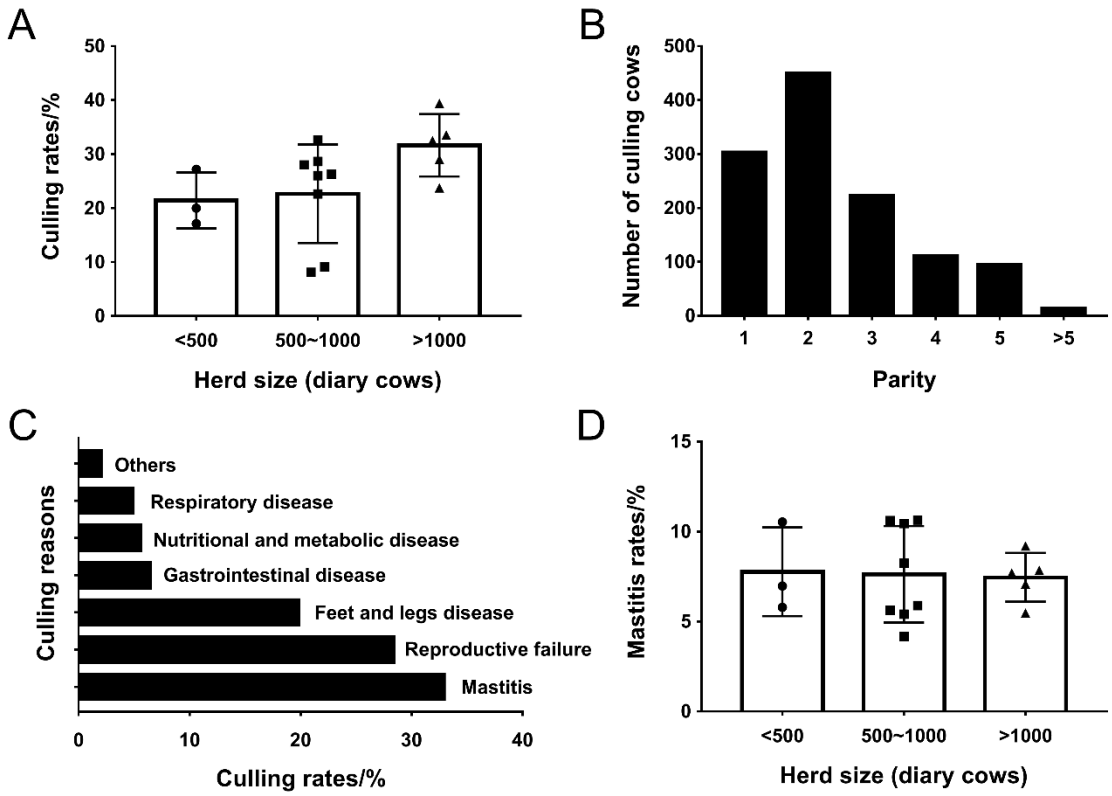


Figure 1 Cow culling rate and reasons in dairy farms. Relation on cow culling rate with herd size and parity (A and B); Difference analysis of culling rates for culling reasons (C); Relation between cow mastitis rates and herd size (D).

Table 1 The identified risk factors associated with clinical mastitis.

Identified risk factors	No. of herd examined	Bovine clinical mastitis		
		Mean proportion (%)	S.D.	*P*
Herd factors				
Herd size				
<500	3	7.72	2.61	
500~1,000	8	7.63	2.66	0.980
>1,000	5	7.41	1.47	
Filler material in cow bed				
Sandy soil	4	5.23	0.74	
Poudrette	4	6.47	0.95	0.000
Fermentation bed	4	8.03	1.1	
Rubber	4	10.58	0.12	
Management factors				
Management of milking parlors				
Excellent managed	6	5.83	0.56	
Good managed	5	6.88	1.57	0.000
Poor managed	5	10.36	0.49	
Cleaning of quarters before milking				
Using disposable wipes	9	6.51	1.95	0.022
Using reusable cloth	7	8.94	1.75	
Disinfection of teat cups every time				
Yes	10	6.62	1.99	0.018
No	6	9.17	1.54	
Cow factors				
Hygiene of breast and leg				
1%~10% contamination	393	7.91	0.33	
10%~30% contamination	393	17.23	2.76	0.027
>30% contamination	393	15.25	1.87	
Nipple score				
Slight injury	393	13.49	1.87	
Moderate injury	393	15.65	1.90	0.008
Severe injury	393	27.61	0.90	

Phylogeny groups and virulence genes of E.coli: All E.coli isolates were collected from cows with symptoms of CM. The majority of the 29 isolates, 18 (62.07%) belonged to phylogenetic groups A, followed by group D (n = 7, 24.14%), B2 (n = 3, 10.34%) and B1 (n = 1, 3.45%). Among the 18 detected virulence genes, the gene iucD (aerobactin) was the most common and was detected in 37.93% (n = 11) of the PCR-positive isolates (Table 2 and Supplementary File 1), followed by genes iss (increased serum survival, n = 10, 34.48%), cnf2 (cytotoxic necrotizing factor 2, n = 3, 10.34%), papC (P-fimbria, n = 1, 3.45%) and sfaD (S-fimbria, n = 1, 3.45%). Moreover, the results indicated that there were no significant differences in the distribution of any of the virulence genes between the phylogenetic groups (Table 2). Of the 29 E.coli isolates, 12 (41.38%) had at least one virulence gene detected by PCR (Table 3). The combinations of virulence genes varied greatly, with between 1 and 4 virulence genes detected per isolate. The gene iucD was the most common and was detected in 91.67% (n = 11) , followed by genes iss (n = 10, 83.33%), cnf2 (n = 3, 25%), papC (n = 1, 8.33%) and sfaD (S-fimbria, n = 1, 8.33%). (Table 3). Genes cva, vat, tsh, irp2, astA, stx1, stx2, eaeA, ehxA, saa, f17A and afa8E were not found in any of the isolates.

Antimicrobial resistance of E.coli isolates: The E.coli isolates were tested for resistance to a panel of 7 most commonly used antimicrobial compounds including amoxicillin, ceftriaxone, ciprofloxacin, doxycycline, gentamicin, levofloxacin, and lincomycin. All of the 29 E.coli isolates were classified as resistant to one or more antimicrobials (Fig.2A). The most frequent resistances were for ciprofloxacin (29/29, 100%), levofloxacin (29/29, 100%), doxycycline (18/29, 62.07%), gentamicin (15/29, 51.72%), lincomycin (11/29, 37.93%). The distribution of antimicrobial-resistant isolates among the phylogenetic groups is shown in Table 4. The results showed that there was no relationship between antibiotic resistance and phylogenetic group. Multi-drug resistance, defined as resistance to three or more antimicrobials, was observed in 18/29 isolates (62.07%) (Fig.2B).

We further assessed the carriage of extended-spectrum β-lactamase genes (bla_{CTX-M}, bla_{TEM} and bla_{SHV-1}) and macrolide resistance genes (erm B, erm C and erm F) and found that the most commonly carried resistance

gene was ermF (37.93%, 11/29). Of the 29 E.coli isolates, the three isolates which were resistant to ceftriaxone were positive for bla_{CTX-M} and bla_{TEM} genes, which encode enzymes capable of degrading β-lactam antimicrobials (Fig.2C). The sequencing of the quinolone resistance-determining region of these genes in highly fluoroquinolone-resistant mutants revealed the presence of gyrA mutations at codons corresponding to Ser-83 and Asp-87 (7/10, Fig.2D). No regular mutations were found in the gyrB, parC, and parE genes.

Table 2 Prevalence of virulence genes among the phylogenetic groups for 29 *E.coli* isolates isolated from bovine clinical mastitis.

Virulence gene	Positive isolates (n=29)		Phylogroup A (n=18)		Phylogroup B2 (n=3)		Phylogroup D (n=7)	
	No.	%	No.	%	No.	%	No.	%
iucD	11	37.93	4	22.22	2	66.67	5	71.43
iss	10	34.48	4	22.22	2	66.67	4	57.14
cnf2	3	10.34	2	11.11	1	33.33	0	0.00
papC	1	3.45	1	5.56	0	0.00	0	0.00
sfaD	1	3.45	0	0.00	1	33.33	0	0.00

Table 3 Different combinations of virulence genes detected among *E.coli* isolated from bovine clinical mastitis (n=29).

Detected virulence genes and their combinations among mastitis isolates				No. of isolates
cnf2	*iucD*			1
cnf2	*iss*	*papC*		1
cnf2	*iss*	*iucD*	*sfaD*	1
iucD				1
iucD	*iss*			8
Total number				12

Table 4 Antimicrobial resistance and prevalence of antimicrobial resistance among the phylogenetic groups for 29 *E.coli* isolates.

Antimicrobial	Phylogroup A (n=18)		Phylogroup B1 (n=1)		Phylogroup B2 (n=3)		Phylogroup D (n=7)	
	No.	%	No.	%	No.	%	No.	%
Amoxicillin	0	0.00	0	0	0	0.00	1	14.29
Ceftriaxone	2	11.11	0	0	0	0.00	1	14.29
Ciprofloxacin	18	100.00	1	100	3	100.00	7	100.00
Doxycycline	12	66.67	0	0	3	100.00	3	42.86
Gentamicin	9	50.00	0	0	3	100.00	3	42.86
Levofloxacin	18	100.00	1	100	3	100.00	7	100.00
Lincomycin	6	33.33	0	0	2	66.67	3	42.86

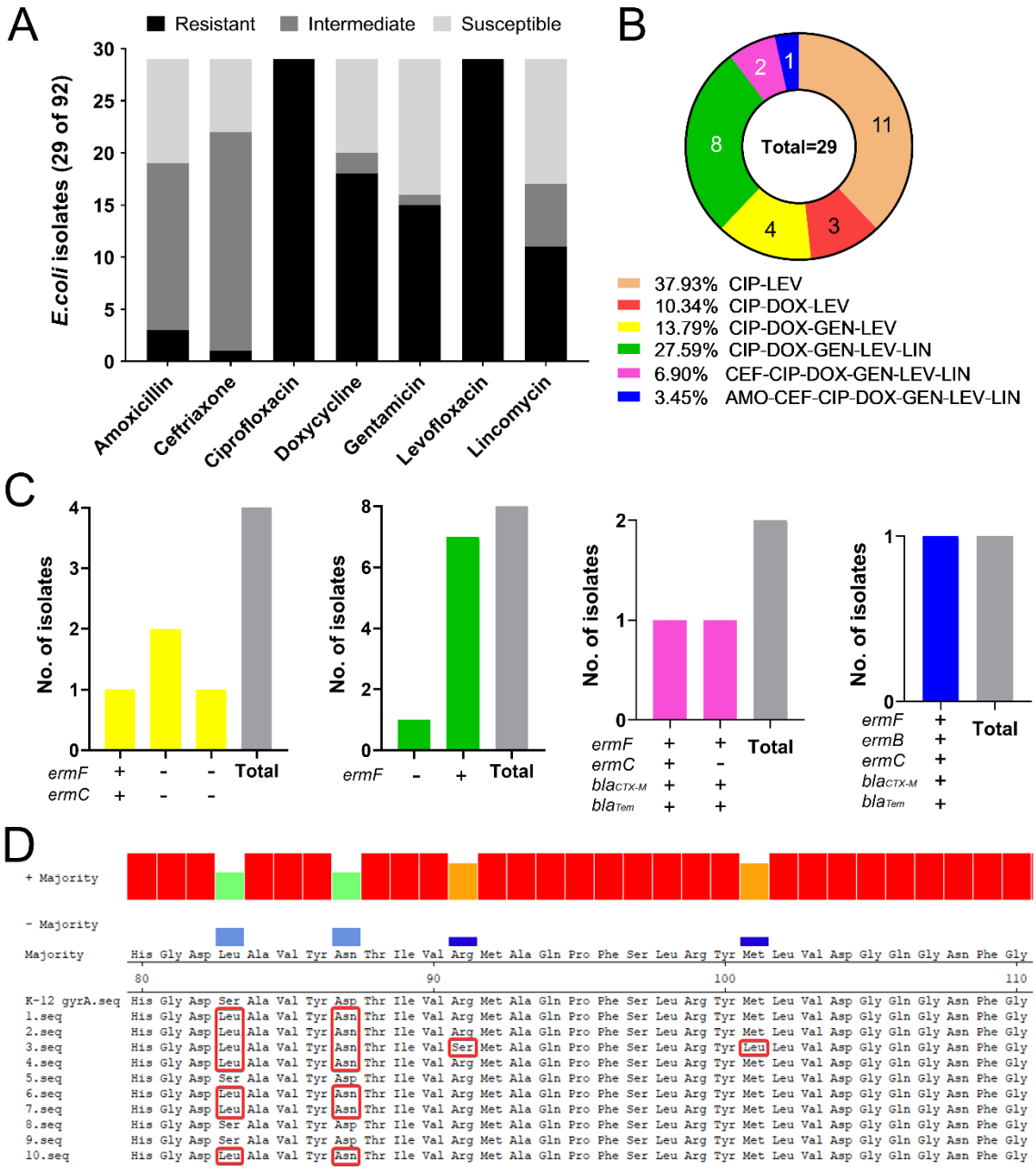


Figure 2 The detection of antimicrobial resistance in E.coli isolates. The E.coli isolates were tested and classified (A); Antibiotic resistances were analysed for ciprofloxacin, levofloxacin, doxycycline, gentamicin, lincomycin (B); Resistance genes were assessed (C). The sequencing of the quinolone resistance-determining region of these genes were analysed (D).

Discussion

In order to achieve high growth of milk yield, cows have experienced a long-term selection and intensive use for milk yield. However, an increased frequency of early involuntary culling has occurred in the current high-yield dairy cattle, which can be attributed to poor animal welfare. During the study period, the average culling rate per year was 25.24%, this is similar to the estimated average for the UK (Bell *et al*., 2010), the Netherlands (Nor *et al*., 2014)and Spain (Armengol and Fraile, 2018). We also found that culling rates for first and second parity cows (25.38 and 37.60%, respectively) were significantly higher than third, fourth and after fourth parity cows (18.68, 9.30 and 9.05%, respectively). After the fourth parity, the turnover rate decreased probably because cows showed good production and reproductive performance (Seegers *et al*., 1998). As for the risk factors for culling associated with cows in Shandong province, most of them were involuntary factors, such as mastitis, reproductive failure, feet and leg diseases. Mastitis can not only reduce milk production but also increase culling costs, replacement costs and disease treatment costs (Rollin *et al*., 2015; Aghamohammadi *et al*., 2018). Previous reports showed that mastitis was associated with an increased risk of culling (Heikkila *et al*., 2012). In this study, we found the average mastitis rate of the investigated herds was 7.6%, which made mastitis to be the primary reason for cow culling. The study closely agrees with previous reports (Lehenbauer and Oltjen, 1998), and indicated that

effective prevention and control of mastitis can effectively promote the increase of breeding efficiency.

CM is an economic and welfare problem on dairy farms (Kerslake *et al*., 2018), which is a multifactorial disease that involves an interaction of microorganisms with host management factors. According to previous reports, herd factors, management factors and cow factors were found to be responsible for the occurrence of CM, while mastitis-associated risk factors differ greatly between geographical areas (Adamczyk *et al*., 2017). These risk factors were selected based on the articles. A similar relationship between the herd size and the length of cow productive life was also found by Jankowska *et al.* (Jankowska *et al*.). In this study, the primary cause of reduced longevity in dairy cows is mastitis. Hence, we speculated that dairy farm size may affect the incidence rate of mastitis. Furthermore, according to the study (Bhandari *et al*., 2021), we select filler material in the cow bed (the nature of the housing) and cleaning of quarters before milking (udder washing before and after milking). In this study, A questionnaire was administered to farmers to identify the risk factors associated with the occurrence of Subclinical mastitis in cows. Finally, the remaining factors, the management of milking parlors (Elmoslemany *et al*., 2010), disinfection of teat cups every time (Elmoslemany *et al*., 2010), hygiene of breast and leg (Dohmen *et al*., 2010; Hohmann *et al*., 2020), and nipple score (De Vliegher *et al*., 2003), were closely related to the infection of mastitis in cows. In our study, we found that herd size did not impact the incidence rate of CM, which was in inconsistent with other researchers who found that smaller herds tended to have a higher incidence of CM(Simensen *et al*., 2010). The risk of mastitis and culling rate were less for sandy soil, which was consistent with the finding that softer floorings were associated with decreased incidence of CM, teat lesions, and cow removal (Ruud *et al*., 2010; Yajima *et al*., 2017).

The current mastitis control programs include hygienic milking, improvement of housing conditions, routine machine maintenance, post-milking nipple disinfection, dry cow treatment, isolation of infected cows, and slaughter of cows (Kelly *et al*., 2009a; Kelly *et al*., 2009b). Some control strategies aim to prevent bacterial transmission between cattle, while others reduce the risk of bacterial infection. It can be seen that the control of bacterial infection has become a key point in the prevention and control of CM. We found that *E.coli* and Streptococcus are the predominant bacteria associated with CM in Shandong province. However, some researchers reported *Staphylococcus aureus* and *Streptococcus uberis* were the most commonly isolated mastitis-causing pathogens (Keane *et al*., 2013; Hoekstra *et al*., 2020; O'Dea *et al*., 2020). Our data are derived from a limited geographical area and farms, which may partly explain the difference compared with national data. Previous reports showed that phylogenetic groups A and B1 were associated with commensal strains of *E. coli*, while B2 was associated with extra-intestinal pathogenic *E. coli* (Son *et al*., 2009). In this study, 62.07% of the isolated E.coli belonged to phylogeny group A, which has been reported to have few virulence factors (Dogan *et al*., 2006; Suojala *et al*., 2011). This agrees with a number of other studies in which the mastitis-causing *E. coli* are typical commensals (Suojala *et al*., 2011; Zhang *et al*., 2018). These results suggested that the infection may be mainly caused by opportunistic infection caused by environmental microorganisms when the host's immunity is low. Therefore, in the face of *E. coli* challenge, cow-specific factors seem to be more important to maintain optimal udder health (Cheng and Han, 2020). In the present study, no bacteria were recovered from 23.76% (24/101) of milk samples from cases of CM. The culture-negative rate reported here is in accordance with previous reports(Lam *et al*., 1996; Garmo *et al*., 2010). At the quarter level, approximately 10-40% of milk samples from cases of CM may yield no growth because of several reasons (i.e., too few or no bacteria present or pathogens that require special culture techniques) (Lam *et al*., 2009).

In this study, 58.62% of the *E.coli* isolates were lack of the detected virulence genes, indicating that many of the strains were opportunistic pathogens(Zhang *et al*., 2018). The combination of virulence genes varied greatly, which indicated that high genotypic variability is characteristic of E.coli strains causing CM(Suojala *et al*., 2011). Of the virulence genes detected here, iucD was the most common and detected in 91.67% of the isolates, followed by iss (83.33%). It was reported that iucD was associated with iss and may be located on either a chromosome or a plasmid, where many of the antimicrobial resistance genes also reside(Johnson *et al*., 2002; Suojala *et al*., 2011). Here, all of the *E.coli* isolates were classified as resistant to one or more antimicrobials that have been used for years in dairy cattle in China. Among the *E. coli* isolates tested, resistance to ciprofloxacin or levofloxacin was most common (100%). We reported a significantly higher occurrence of quinolone-resistant E.coli from bovine mastitis cases, as compared with previous reports (22.4-25%) about dairy cattle suffering mastitis in China(Wang *et al*., 2008; Zhao *et al*., 2014; Yang *et al*., 2018). The main mechanisms of quinolone-resistance in bacteria have been attributed to several point mutations in the gyrA and parC genes (Röderova *et al*., 2016). In the present study, we found that seven isolates (70%) had double mutations (Ser83Leu/Asp87Asn) in the gyrA gene. These results suggest that gyrA gene mutation may be one of the reasons for the high resistance of E.coli to quinolones from bovine mastitis cases in China, while the specific mechanisms should be studied further.

More importantly, 62.07% of the isolates showed multi-drug resistance(MDR) in our study, which was significantly higher than the percentage observed from the surveillance data in ten provinces of China (Cheng *et al*., 2019). However, it is lower than the 100% multi-drug resistance rate reported by Yu *et al*. (2020) (Yu *et al*., 2020), which indicates that mastitis-causing E.coli in China shows different levels of MDR, and the MDR varies greatly in different provinces. The resistance of mastitis-causing E.coli may reflect the widespread use of antimicrobials in cattle, as these bacteria are commensals. Our results suggest that it is necessary to continue to monitor the drug resistance of E.coli isolates in order to determine the appropriate prevention and control measures for cows with CM. More importantly, it is necessary to study the

mechanisms underlying the antimicrobial resistance in these isolates.

The primary cause of the emergence of MDR strains is the overuse of antibiotics. At present, cow mastitis treatment still depends on traditional antibiotics (Rainard *et al*., 2018; Smulski *et al*., 2020), but this method is easy to produce drug resistance, resulting in the emergence of multi-drug resistant strains (Peng *et al*., 2022). Resistance to antimicrobials such as penicillin, amoxicillin, tetracycline, Amikacin, gentamicin, or erythromycin has been widely reported (Peng *et al*., 2022).

Measures to prevent infections by MDR strains include: (1) Reducing the use of antibiotics. It is necessary to decrease the usage or find alternatives to gradually address the issue of antibiotic resistance. Currently, commonly used alternatives include antimicrobial peptides, traditional Chinese medicine, and probiotics. A study revealed that NZ2114 (Antimicrobial peptide) alleviated mammary gland inflammation, inhibited bacterial proliferation, and reduced the number of mammary gland bacteria (Yang *et al*., 2022). FTA is one of the main effective components of Forsythia suspensa, which has a Traditional Chinese Medicine, and has broad application prospects in the prevention and treatment of bovine mastitis (Liu *et al*., 2024). In another study, *Bacillus subtilis* C-3102 can serve as an alternative to antibiotics to prevent mastitis, and the use of probiotics in this way can lessen the risk of antibiotic-resistant bacteria developing(Urakawa *et al*., 2022). (2) Farms should enforce strict guidelines for staff to promptly clean manure from barns and exercise areas, ensure proper disinfection, and maintain leveled bedding. These actions can improve barn conditions and reduce the risk of mastitis caused by bacterial infections. (3) Implementing enhanced scientific management practices in the milking parlor is also crucial.

In summary, we reported the incidence of cow culling and the associated reasons in Shandong province. We found that mastitis is the main reason for the culling of dairy cows, and the occurrence of mastitis is closely related to the level of feeding and management, equipment and health conditions of cows. In this study, *E.coli* is the main pathogenic bacteria of CM. The results showed that most of these isolates belong to group A, and most of them carry virulence genes of iucD and iss. In addition, the *E.coli* isolates were resistant to one or more antimicrobials that have been used in dairy cattle, and the resistance to ciprofloxacin and levofloxacin was most prevalent among the isolates. The mutations in gyrA may contribute to the high-level of quinolone resistance. Our findings indicate that it is imperative to improve the equipment and sanitary conditions of dairy farms and use antimicrobials rationally, and continue monitoring the prevalence of the antimicrobial resistance in *E.coli* isolates.

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