SHORT REPORT

Identification and histopathological analysis of cow acute mastitis caused by *Escherichia coli* and screening of sensitive drugs: a case study

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Cow mastitis is one of the three major diseases endangering dairy farming, which is mainly caused by pathogenic microorganisms. *Escherichia coli* infection is generally at high level before and after delivery and early lactation of dairy cows. This study reported an acute cow mastitis case 185 days after lactation, which was about the middle lactation period. The mastitis of diseased cow was initially diagnosed by the observation of clinical symptoms, and then, confirmed by autopsy performed by a certified veterinary doctor. In order to determine the etiology of a cow suffering from acute mastitis in a cattle farm, the pathogen was isolated and identified from the mammary glands of diseased cow. Mammary gland tissue and milk from the diseased cow were inoculated on 5% sheep blood agar plates and Eosin methylene blue agar to isolate the bacterial strains. The genomic DNAs of the isolates were extracted and 16S rRNA genes were amplificated and sequenced. After bacterial isolation, identification, and 16S rRNA sequencing analysis, the acute mastitis of diseased cow caused by *Escherichia coli* was determined. A large amount of eosinophilic exudate, necrosis and nuclear fragmentation of parenchymal cells, and multiple calcifications were observed in the mammary gland tissues of diseased cows. Through the sensitive drug screening, the isolated bacterial strain was highly sensitive to gentamicin, spectinomycin, kanamycin, chloramphenicol, doxycycline, and tetracycline hydrochloride, while it was moderately sensitive to cefoxitin. The bacterial strain was resistant to compound sulfamethoxazole, ampicillin, and cefotaxime. The results of this study provided reliable data reference for the targeted prevention and treatment of mastitis in the cattle farm.

Keywords: cow; mastitis; histopathological analysis; *Escherichia coli*; sensitive drug screening.

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Introduction

Cow mastitis is one of the three major diseases endangering dairy farming, which is mainly caused by pathogenic microorganisms [1]. At present, it is found that there are many kinds of pathogenic microorganisms causing dairy cow acute mastitis, including more than 200 kinds of bacteria, fungi, viruses, and mycoplasma. Among which, bacterial infection is the main factor [2]. *Staphylococcus aureus*, *Streptococcus*, and *Escherichia coli* are three main bacteria causing mastitis in dairy cows [3-5]. *Escherichia coli* (*E. coli*) is a representative bacterium of the *Escherichia spp*. and is the primary pathogenic bacteria of clinical cow mastitis. It widely exists in

the external environment and can invade cow breast tissue through a variety of ways to trigger inflammatory reaction, leading to cow mastitis. Cow mastitis is caused by a variety of factors, mainly characterized by breast enlargement, pain, decreased milk production, and milk denaturation, which affects the development of the world dairy industry and causes huge losses to the production of dairy products [6].

In livestock production, antibiotics are often used for the prevention and treatment of mastitis. However, the irrational use, especially abuse, of antibiotics has led to the widespread existence of drug-resistant bacterial strains and even multi drug resistant strains, which has not only seriously affected the healthy development of animal husbandry, but also brought serious harm to public health safety [7].

In order to determine the causes of cow mastitis, this study collected the pathological materials of diseased cow to isolate and identify pathogens. In addition, through the analysis of 16S rDNA gene sequence, histological changes, and antimicrobial susceptibility, the results of this study provided a scientific basis for the prevention and control of this disease.

Materials and methods

Sample collection

In August 2022, a 4-year-old cow from Anyixin Farming and Animal Husbandry Co. LTD (Wuzhong, Ningxia, China) developed acute mastitis and died just few hours after the disease was onset. The subject was healthy and had been lactating for 185 days with the average milk production of 61 L per day before the onset of disease. The mastitis was initially diagnosed by the observation of clinical symptoms, and then, autopsy by a certified veterinary doctor. The mammary glands and milks of diseased cow were collected in sterile sample tubes, and then, were transported to the Clinical Veterinary Laboratory at Ningxia Academy of Agriculture and Forestry Sciences (Yinchuan, Ningxia, China) within 2 hours after collections for etiological diagnosis and histopathological studies.

Bacteria isolation and culture

Mammary gland tissue of diseased cow and milks were inoculated on 5% sheep blood agar plates (Lab-Lemco powder 10 g/L, peptone neutralized 10.0 g/L, sodium chloride 5.0 g/L, agar 15.0 g/L, and defibrinated sheep blood) (Oxoid, Basingstoke, Hampshire, UK) and Eosin methylene blue agar plates (Haibo, Qingdao, Shandong, China), respectively, and were cultured in BSD-YX3200 constant temperature incubator (BoXun, Shanghai, China) at 37°C for 24 hours. The isolated bacterial colonies were inoculated into the Tryptic Soy Broth (tryptone 17.0 g/L, soy peptone 3.0 g/L, sodium chloride 5g/L, K_2HPO_4 2.5 g/L, glucose 2.5 g/L) (Oxoid, Basingstoke, Hampshire, UK) according to the colony morphology, and were cultured in QYC-200 bacterial culture oscillator (Yiheng, Shanghai, China) at 37°C, 220 rpm for 12 hours [8].

DNA extraction and polymerase chain reaction (PCR)

Two milliliters of bacterial culture (> 1×10^8 Colony Forming Unit (CFU)/mL) were centrifuged by using Eppendorf 5418R centrifuge (Eppendorf, Hamburg, German) at 12,000 rpm for 2 mins to obtain the precipitation of the bacterial strains. The genomic DNAs of isolated bacterial strains were extracted by using TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0 (Takara, Osaka, Osaka Prefecture, Japan). The 16S rRNA genes were amplified by using BIO-RAD S1000 thermal cycler (Bio-Rad, Hercules, California, USA) and TaKaRa 16S rDNA Bacterial Identification PCR kit (Takara, Osaka, Osaka Prefecture, Japan) with the forward primer of 5'-GAA TTC CGA GAG TTT GAT CCT GGC T -3' and reverse primer of 5'- AAG CTT GAG GTA ATC CAT CCC CAC GTT C -3'. The PCR reaction mixture was 50 µL with 2 µL of template DNA, 25 µL of PCR Premix, 1 µL of forward primer, 1 µL of reverse prime, 21 µL of H_2O. The reaction was performed at 94°C for 5 mins, followed by 30 cycles of 94°C

for 1 min, 50°C for 1 min, and 72°C for 1.5 mins, then 72°C for 5 mins. The PCR products were analyzed on 1% agarose gel electrophoresis, and then, were purified by using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0 (Takara, Osaka, Osaka Prefecture, Japan). The purified PCR products were sent to Shenggong Bioengineering Co., Ltd (Shanghai, China) for sequencing [8, 9].

Phylogenetic analysis

The sequencing data were evaluated to determine the closest relatives by using nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). A phylogenetic analysis of sequences together with sequences of the closest relatives available in the GenBank (https://www.ncbi.nlm.nih.gov/) database was conducted by using the neighbor-joining (NJ) method and Kimura 2-parameter model in the MEGA 6.0 software (DNAStar, Madison, Wisconsin, USA). Bootstrap confidence values (1,000 replications) were given at the respective nodes [9, 10].

Histological observation

Mammary gland tissues of diseased cow were immersed in 10% neutral formaldehyde solution fixed for 7 days. The fixed tissues were dehydrated by the following sequences as 75% ethanol for 4 hours, 85% ethanol for 2 hours, 95% ethanol for 1 h, 100% ethanol for 0.5 h for 4 times, xylene for 10 min twice, Paraffin wax for 1 h, paraffin wax for 2 hours, paraffin wax for 3 hours in JT-12S automatic dehydrator (Junjie, Wuhan, Hubei, China). The BMJ-A embedding machine (Zhongwei, Changzhou, Jiangsu, China) was used to embed tissues. The Leica-2016 rotary microtome (Leica Microsystems, Wetzlar, Germany) was applied for sectioning, while the RS36 automatic staining machine (Paisijie, Changzhou, Jiangsu, China) was used for staining of tissues section following the procedures of dewaxing, hematoxylin staining for 10-20 mins, rinsing with water for 1-3 mins, hydrochloric acid alcohol differentiation for 5-10 s, rinsing with water for 1-3 mins, immersing in warm water (50°C) or weakly alkaline aqueous solution until blue appears, rinsing with water for 1-3 mins, adding 85% ethanol for 3-5 mins, eosin staining for 3-5 mins, washing with water for 3-5 s, gradient ethanol dehydration, xylene transparent, and neutral gum sealing. A digital slice scanner (3DHISTECH, Budapest, Hungary) was used to collect images of the slices [9].

Antimicrobial susceptibility tests

According to Clinical and Laboratory Standards Institute (2018) Performance for Antimicrobial Susceptibility Testing (28th Edition, Clinical and Laboratory Standards Institute, Wayne), the colony suspension was equivalented to a 0.5 McFarland standard by using Kirby-Bauer method. In aseptic operation, the colony suspension was evenly coated on Mueller-Hinton agar (MHA) with 5% sheep blood. After the surface of the medium was dried, the antimicrobial sensitive paper was placed on the surface of the culture medium, and the diameter of the bacteriostatic zone was measured after being cultured in a constant temperature incubator at 37°C for 18-20 hours [9]. *Escherichia coli* ATCC 25922 (GenBank ID: CP009072.1) was used as the quality control strain. Each antimicrobial susceptibility test was repeated three times. The criteria for the interpretation of zone diameter used in this study were described in Table 1.

Results

Observation of collected samples

The subject diseased cow suffered from hemorrhage in the left front and rear mammary glands (Figure 1-A1), and a small amount of congestion in the right front and rear mammary glands (Figure 1-B1). The milk secreted by the left breast was black with blood (Figure 1-A2), and the milk secreted by the right breast was white (Figure 1-B2).

Table 1. Zone of inhibition diameter interpretive criteria for *Enterobacteriaceae*.

Antibiotic family	Antibiotics	Abbreviation	Disc content	R (mm)	I (mm)	S (mm)
Aminoglycosides	Gentamicin	C	10	≥ 15	13–14	≤ 12
	Streptomycin	S	10	≥ 15	12–14	≤ 11
	Kanamycin	K	30	≥ 18	14–17	≤ 13
Phenicols	Chloramphenicol	C	30	≥ 18	13–17	≤ 12
Sulfonamides	Sulfamethoxazole	SXT	25	≥ 16	11–15	≤ 10
Tetracyclines	Doxycycline	DO	30	≥ 14	11–13	≤ 10
	Tetracycline	TE	30	≥ 15	12–14	≤ 11
	Ciprofloxacin	CIP	5	≥ 21	16–20	≤ 15
	Ofloxacin	OFX	5	≥ 16	13–15	≤ 12
	Levofloxacin	LEV	5	≥17	14-16	≤13
β-lactams	Ampicillin	AMP	10	≥17	14-16	≤13
	Cefotaxime	CTX	30	≥ 26	23–25	≤22
	Cefoxitin	FOX	30	≥ 18	15–17	≤14

Notes: R: resistant, **S:** susceptible, **I:** intermediate.

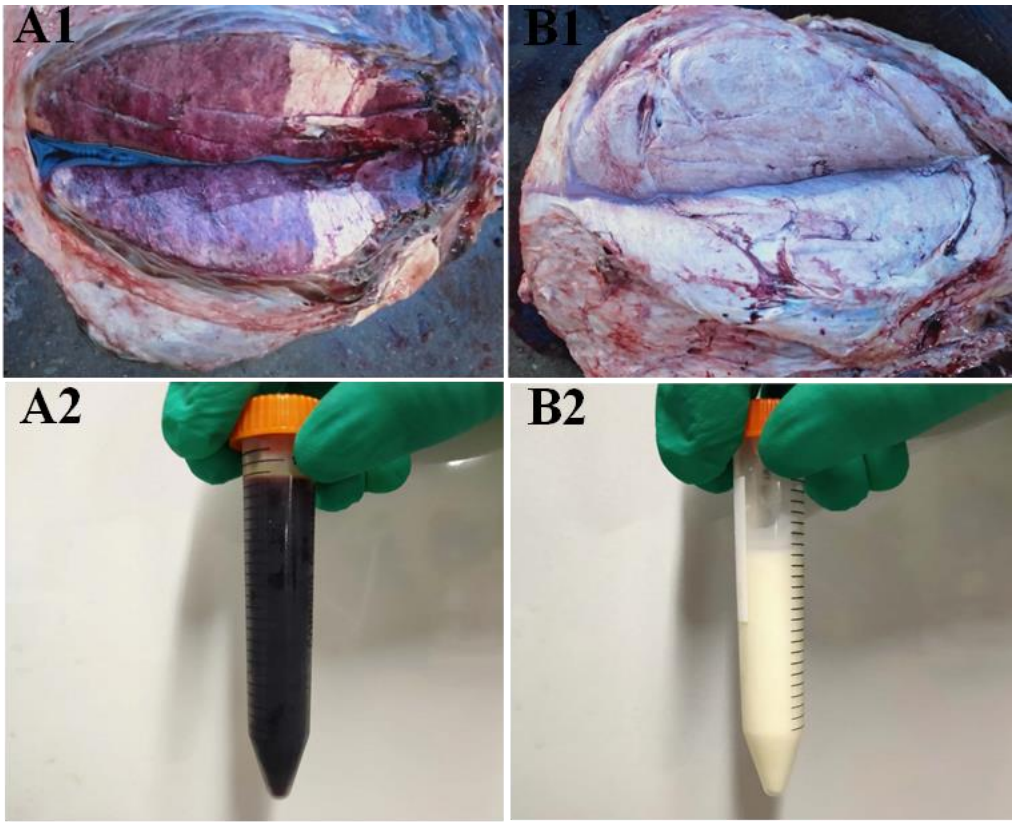

Figure 1. Histopathological changes in mammary glands and milk of diseased cow.

Observation results of colony morphology

After 24 hours culturing of tissue samples on the solid medium of defibrated sheep blood and Eosin methylene blue agar at 37°C, the single colony that was medium gray round was formed on the blood plate, while the single colony with metallic luster was observed on Eosin methylene blue agar plate. Gram staining and microscopic

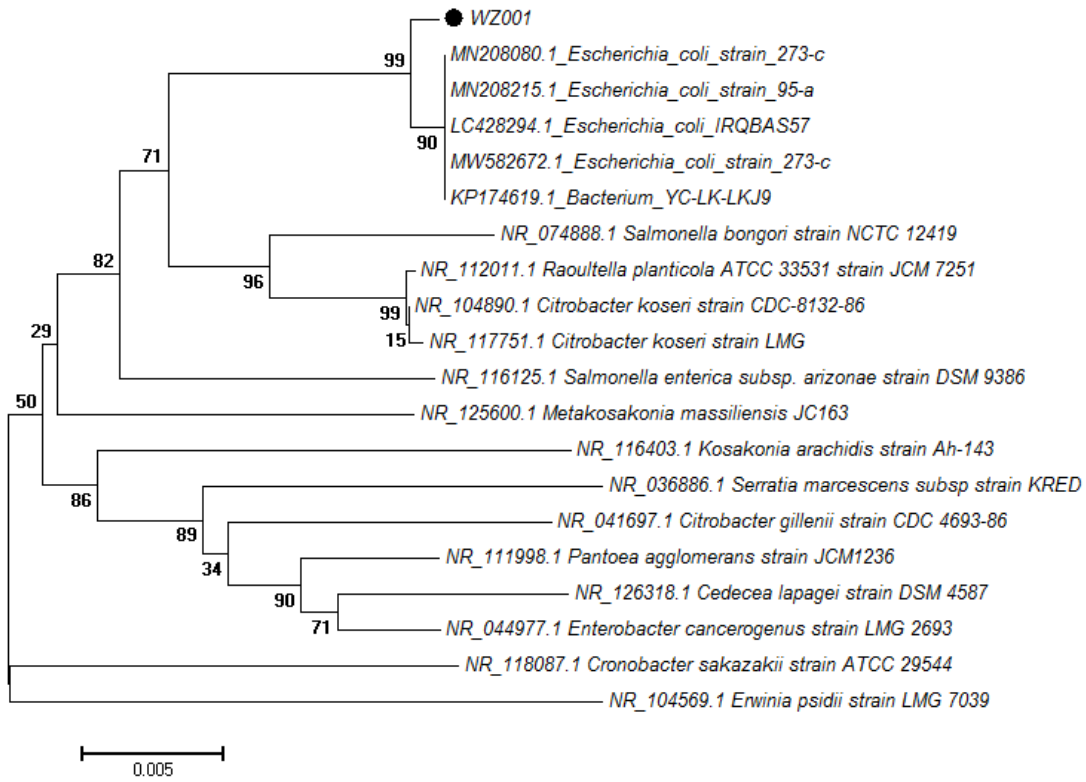

Figure 2. Phylogenetic tree of isolated strain was constructed based on 16S rRNA gene sequence.

examination showed that the bacterial strain was Gram negative and bacillus brevis with blunt round ends, which was named WZ001.

PCR identification and phylogenetic analysis

The PCR amplified 16S rRNA products showed that the length of the amplified fragment was about 1,500 bp. The phylogenetic tree analysis demonstrated that the isolated bacterial strain WZ001 was on the same branch as *Escherichia coli* strain 273-c (GenBank ID: MN208080.1), 95a (GenBank ID: MN208215.1), and IRQBAS57 (GenBank ID: LC428294.1). It was distantly related to *Salmonella bongori*, *Raoultella planticola*, *Citrobacter koseri*, *Salmonella enterica*, *Metakosakonia massiliensis*, *Kosakonia arachidis*, *et al*. (Figure 2).

Histological observation

A large amount of eosinophilic exudate, necrosis and nuclear fragmentation of parenchymal cells, and multiple calcifications were observed in the mammary gland tissues of diseased cow (Figure 3).

Antimicrobial susceptibility tests

The results of antimicrobial susceptibility analysis of *E. coli* to 13 antibiotics were shown in Table 2. The isolated strain WZ001 was highly sensitive to Gentamicin, Streptomycin, Kanamycin, Chloramphenicol, Doxycycline, Tetracycline, Ciprofloxacin, Ofloxacin, Levofloxacin, while it was moderately sensitive to Cefoxitin. It was resistant to Sulfamethoxazole, Ampicillin, and Cefotaxime.

Discussion

Cow mastitis is a major production limiting disease in dairy industry worldwide [11-13]. In addition to the negative impact on animal welfare and the farm economy, the widespread use of antibacterial agents to treat and manage

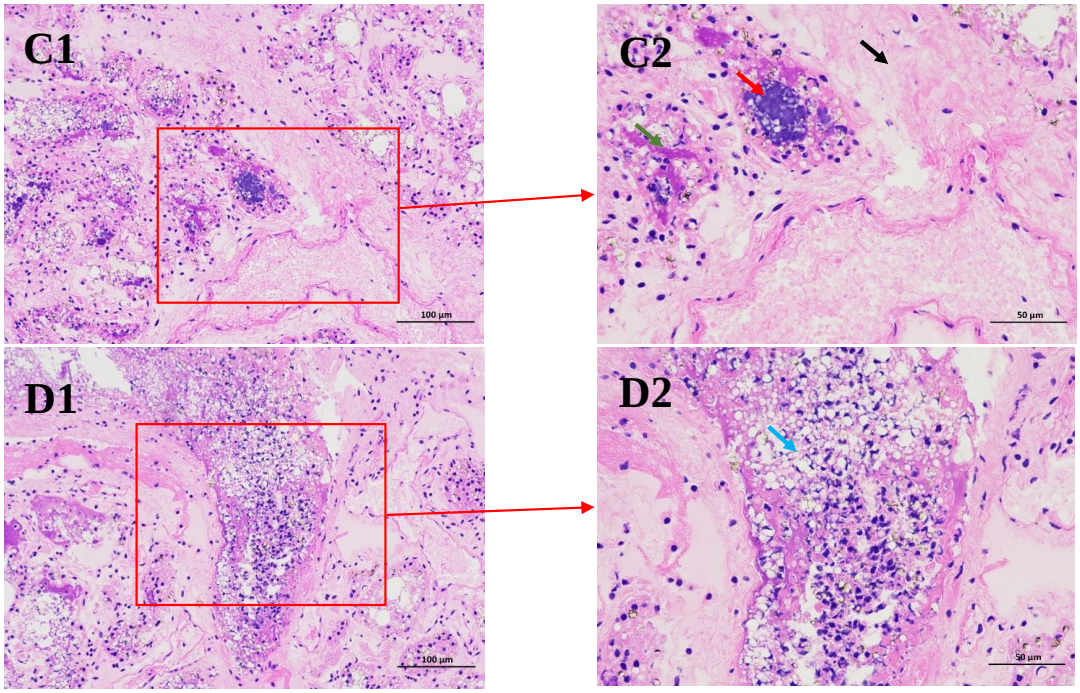

Figure 3. Histological observation results (H.E. staining). **C1 (200×) and C2 (400×):** a large number of eosinophils exuded from the tissues (black arrow), multiple calcifications (red arrow), and tissue bleeding (green arrow). **D1 (200×) and D2 (400×):** substantial cell necrosis and nuclear fragmentation (blue arrow).

Table 2. Antimicrobial susceptibility results.

Antibiotic family	Antibiotics	Abbreviation	*E. coli* (WZ001)	Susceptibility
Aminoglycosides	Gentamicin	C	27.33±0.47	S
	Streptomycin	S	26.00±0.00	S
	Kanamycin	K	25.00±0.00	S
Phenicols	Chloramphenicol	C	22.67±0.47	S
Sulfonamides	Sulfamethoxazole	SXT	7.00±0.00	R
Tetracyclines	Doxycycline	DO	22.67±0.47	S
	Tetracycline	TE	22.00±0.82	S
	Ciprofloxacin	CIP	22.67±0.47	S
	Ofloxacin	OFX	20.00±0.82	S
	Levofloxacin	LEV	25.00±0.82	S
β-lactams	Ampicillin	AMP	10.00±0.82	R
	Cefotaxime	CTX	15.00±0.00	R
	Cefoxitin	FOX	17.67±0.47	I

mastitis is also a major public health problem [14]. Cow mastitis is an inflammatory change in the breast caused by physical, chemical, microbial, and other pathogenic factors including external mechanical causes such as incorrect milking method, failure to follow operating procedures, unhygienic cowshed, and poor feeding management, which can damage or reduce the physiological resistance of the breast [15].

Cow mastitis can be divided into clinical mastitis and recessive mastitis [16]. Clinical acute mastitis is one of the major diseases threatening the dairy

industry [17]. *Escherichia coli*, which can cause the death of sick cattle, is an environmental pathogen [18]. After invading the mammary gland, the pathogen can reproduce in a large number in a short time, and then, releases endotoxin that enters the blood and causes toxemia [19]. In this study, the round colony with metallic luster on Eosin methylene blue agar was isolated from the breast tissue of dairy cow with acute mastitis, while the shape of the colony on the blood plate was medium sized, gray white, opaque round. The colony was negative on Gram staining. The microscopic examination showed scattered, blunt round at both ends, red short bacilli, which was consistent with the morphology of *Escherichia coli* reported by Luoreng, *et al*. [15]. The results of 16S rRNA sequencing and phylogenetic tree analysis confirmed that the main pathogen causing acute mastitis in dairy cow was *Escherichia coli*. Relevant studies have shown that the infection rate of *Escherichia coli* is generally at high level before and after delivery and early lactation in dairy cows. However, the acute mastitis of cow in this study occurred 185 days after lactation, which belonged to the middle lactation period. The possible cause might be that the cow pen was wet after the rain and there were potholes and ponding. In addition, the feces on the ground surface were accumulated, and the ventral part of the body contacted the dirty surface, which caused *Escherichia coli* in the environment entering the breast tissue for mass reproduction. Therefore, early diagnosis and environmental sanitation are important measures to reduce coliform mastitis.

The irrational use of broad-spectrum antibiotics has led to the emergence of bacterial resistance and even multiple resistance [20]. Therefore, isolation and identification of pathogens, drug sensitivity screening, and targeted use of antibiotics are important means to maintain large-scale cattle breeding and healthy development. In this study, drug sensitivity test showed that the *Escherichia coli* found in the cattle farm had been exposed to sulfa and β-lactams drugs, which caused resistance to the compound sulfamethoxazole and ampicillin and cefotaxime. The resistance might be related to the long-term use of such antibiotics in the dairy farm. Therefore, large-scale dairy farms should regularly carry out pathogen identification and drug sensitivity studies on cows with mastitis, and select a variety of sensitive antibiotics for appropriate drug rotation to treat cow mastitis to reduce the production of drug-resistant bacterial strains.

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