RESEARCH ARTICLE

Isolation, identification, and antibiotic sensitivity screening of pathogens causing calf respiratory disease in a large-scale dairy farm in Ningxia, China

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Calves are prone to respiratory diseases during the autumn and winter due to significant temperature changes that affect host immunity and promote the spread of infectious diseases. This study aimed to identify the major types of respiratory pathogens affecting calves in a large-scale dairy farm in Ningxia, China and to determine their sensitivities to antibiotics through susceptibility tests, thereby, providing a basis for the prevention and treatment of related respiratory diseases in calves. In mid-November 2022, an outbreak of calf respiratory disease occurred across a large-scale cattle farm of 48 calves in Ningxia, China with 20 sick animals. The nasal swabs of diseased calves symptomized with abdominal respiration, cough, and purulent nasal fluid were collected. The pathogens were then isolated and purified by culture. The resulting purified bacterial strains were subjected to 16S rRNA sequencing. The sequencing results were used to conduct homology comparison and genetic evolution analysis. Moreover, the isolated bacterial strains underwent antibiotic susceptibility tests for 27 antibiotics. The results showed that 3 different bacterial strains were isolated from the bacterial purification culture. The homology comparison and genetic tree analysis demonstrated that the isolates FLZ1, FLZ2, and FLZ3 had high sequence homology with *Klebsiella pneumoniae*, *Pasteurella multocida*, and *Streptococcus multianimalis*, respectively. The antibiotic susceptibility tests showed that the three isolates were all sensitive to amikacin, erythromycin, linezolid, enrofloxacin, and chloramphenicol. This study provided a reference for the diagnosis and prevention of current respiratory diseases affecting dairy calves in Ningxia, China.

Keywords: calves; respiratory diseases; *Kleiber pneumoniae*; *Pasteurella multocida*; *Streptococcus multianimalis*; susceptibility testing.

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Introduction

The dairy industry is an important one involving animal husbandry in Ningxia, China [1], and calf health has an important impact on the quality of dairy products [2]. Calves are prone to respiratory diseases during the autumn and winter period due to significant temperature changes that affect host immunity and promote the spread of infectious diseases [3]. Common pathogens that cause respiratory diseases in calves include *Mycoplasma bovis*, *Pasteurella multocida* (*Pm*), *Mannheimia haemolytica* (*Mh*), *Klebsiella pneumoniae* (*Kp*), infectious

rhinotracheitis virus, bovine viral diarrhea virus, and bovine parainfluenza type 3 virus [4]. Among the bacterial pathogens that cause respiratory diseases in calves, *Kp* is a typical environmental pathogen belonging to the *Klebsiella spp*. [5]. *Klebsiella pneumoniae* is ubiquitous in nature and can either directly or indirectly infect the host through the nose or mouth, consequently causing a variety of ailments in dairy cows including respiratory diseases, diarrhea, meningitis, and mastitis [5]. In recent years, there has been a worsening epidemic caused by *Kp* in China's dairy farming industry, and the resulting harm to the dairy farming industry has become an increasingly serious issue [6]. *Pm* is a gram-negative bacterium that can infect a variety of livestock and poultry *via* its transmission through the digestive tract and respiratory tract. *Pm* mostly causes acute epidemics, and the main symptoms of infected animals include decreased appetite, weight loss, edema, and diarrhea, while more severe symptoms can lead to animal death [7]. Data show that China's infected cattle primarily harbor *Pm* type A and *Pm* type B, which can cause severe fibrinous pneumonia and hemorrhagic sepsis. *Pm* type A mainly causes pneumonia in calves, while *Pm* type B mainly causes hemorrhagic sepsis in adult cattle [8]. *Streptococcus pluranimalium* (*Sp*) was first identified by Devriese in 1999 in the reproductive tracts and tonsils of cows suffered from hidden mastitis. It has also been isolated from the canary sac, respiratory tract, and tonsils of sheep and cats [9]. The species is associated with calf meningitis and may be a sequela of sepsis [10]. Numerous clinical cases suggest that this species primarily infects cattle and poultry [11], but multi-species *Streptococcus* infection in dairy calves has been less commonly reported. In this study, nasal swabs were obtained from diseased calves with abdominal respiration, cough and purulent nasal fluid as the clinical symptoms, and the infecting pathogens were isolated and identified prior to antibiotic sensitivity tests. The findings of this study helped to further understand the incidence of respiratory infectious diseases existing in dairy calves in Ningxia, China during the autumn and winter period, such that corresponding treatment suggestions could be more appropriately administered to address the respiratory diseases of calves in this area.

Materials and methods

Collection of samples

In mid-November 2022, an outbreak of calf respiratory disease occurred across Sunjiatan dairy farm (total 48 calves) in Wuzhong City, Ningxia, Hui Autonomous Region, China with 20 sick calves, 0 death, and 28 disease-resistant calves. The incidence rate was 41.67%. The sick calves primarily presented the following symptoms including dry and cracked nasal mirrors, laying down behavior (for the severely ill), breathing in a chest-abdominal style, having the mouth agape, and a high respiratory rate. During the onset period, the feed intake of the sick cattle decreased, in some cases to the point of nil intake. The disease-resistant cattle could gradually resume feeding and drinking water in the later stage of the disease. During the sampling process, it was found that the sick calves had more secretions from the mouth and nose, some had arched backs, and their blood was black. To confirm the presence and identities of the infecting pathogens, sterile cotton swabs were used to collect samples of deep nasal fluid from the nasal cavities of the diseased calves, which were then placed in 2 mL normal saline sterile centrifuge tubes. A total of 5 nasal swabs were collected from each sick calves and were then stored under 4°C before sent to the veterinary laboratory of the Institute of Animal Science, Ningxia Academy of Agriculture and Forestry Sciences (Yinchuan City, Ningxia, China) for pathogenic diagnosis and antibiotic susceptibility testing.

Isolation and purification of the infecting bacteria

The nasal swab samples that were collected from the sick calves were each coated and inoculated onto blood plates (5% defibrinated sheep blood, 38 g of soybean flour agar, dissolved in 1 L of

ultrapure water) and Mycoplasma bovis solid medium agar plates (21 g/L of PPLO broth, 2 g/L of glucose, 2 g/L of sodium pyruvate, 0.6% agarose, 100 mL/L of yeast infusion with a mass volume ratio of 25%, 0.18 mL/L of 0.4% phenol red, 100 mL/L of inactivated horse serum, and 200 IU/mL of water-dissolved penicillin, pH 7.4-7.6). All plates were incubated at 37°C for 16-24 h. A single colony was selected from each plate and the culture was repeated on a blood plate. Each purified colony was inoculated in nutrient broth (10 g/L of Lab-Lemco powder, 10.0 g/L of peptone neutralized, 5.0 g/L of sodium chloride) and was cultured in a constant temperature incubator (BSD-YX3200, BoXun, Shanghai, China) at 37℃, 180 rpm for 24 hours before being stored at -20°C for subsequent use. If the Mycoplasma grew, individual colonies on the Mycoplasma medium were microscopically inoculated into Mycoplasma bovis liquid medium and then cultured at 37°C, 220 rpm for 48 h.

Polymerase chain reaction (PCR) amplification and sequencing of the purified strains

The 16S rDNA universal primers designed by Halium, *et al*. were employed in this study [12] and were synthesized by Shenggong Bioengineering (Shanghai, China) with the sequences as 27F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R: 5'-TAC GGC TAC CTT GTT ACG ACT T-3'. 2 mL of the liquid culture of each bacterial strain was centrifuged at 12,000 rpm for 2 min before the genomic DNA extraction by using bacterial genomic DNA extraction kit (Tiangen, Beijing, China). The PCR reaction was set as 25 µL of total volume with 12.5 µL of 2× TaqPCR Master Mix, 1 µL of 20 µmol/L forward and reverse primers, 2 µL of genomic DNA template, and 9.5 µL of ddH_2O, and performed under the program of 95°C for 2 min followed by 30 cycles of 95℃ for 30 s, 53℃ for 20 min, and 72°C for 2 min, and then, 72℃ for 8 min. by using a thermocycler (BIO-RAD, California, USA). The estimated amplification fragment was about 1,465 bp. The PCR amplification products were then separated by 1% gel electrophoresis at 120 V for 30 min. The target fragment DNAs were purified and recovered from the gel by using a gel recovery kit (OMEGA, Georgia, USA). The purified PCR products were then sent to Shenggong Bioengineering (Shanghai, China) for sequencing.

Phylogenetic Analysis

The sequencing results of the isolated bacterial strains were spliced by using LaserGene software (DNAstar, Madison, Wisconsin, USA). The splicing sequences were compared by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree of the isolated bacterial strains was constructed based on the sequences of 16S rDNA by using the adjacency method, Neighbor-joining (NJ), in MEGA 7.0 software (DNAstar, Madison, Wisconsin, USA), and the genetic evolution was analyzed.

Antibiotic sensitivity screening for the isolated bacterial strains

27 antibiotics were selected to conduct susceptibility tests on the isolated bacterial strains. For each bacterial strain, 100 µL of bacterial solution was mixed with 900 µL of saline. The OD_{600} value was adjusted to 0.10-0.12 by using IMPLEN N60 (Implen, Munich, Germany) before being dipped in a disposable sterile cotton swab and applied to an MH agar plate (1.5 g/L soluble starch, 13.0 g/L agar powder, 5.0 g/L beef paste powder, 17.5 g/L hydrolyzed casein). After the surface of the medium was dried, the antibiotic paper was pasted on the surface of the culture medium, and the diameter of the bacteriostatic zone was measured after the plate being cultured in a constant temperature incubator at 37℃ for 18-20 hours. Antibiotic sensitivity was determined by reference to the Clinical and Laboratory Standards Institute (2018) Performance for Antimicrobial Susceptibility Testing (28th Edition, Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA).

Results

Bacterial strain isolation

No Mycoplasma growth was observed in the Mycoplasma bovis medium. There were three different forms of colonies observed on the blood

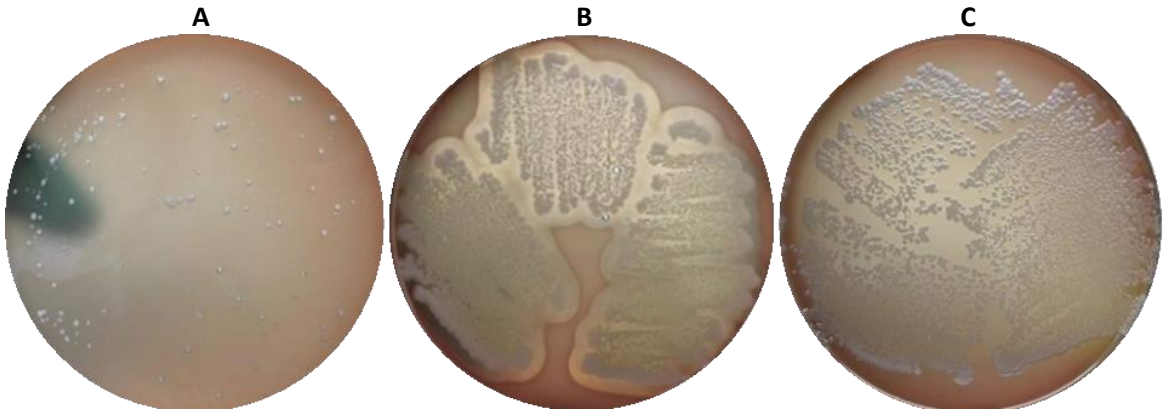

Figure 1. Photographs of the three purified colony types. **A.** white and rounded, milky-white in the middle and gray-transparent on the outside. **B.** grayish-white, moist, sticky, and a clear hemolytic ring around the colonies. **C.** grayish-white, smooth, dry appearance, and observable reflection under light.

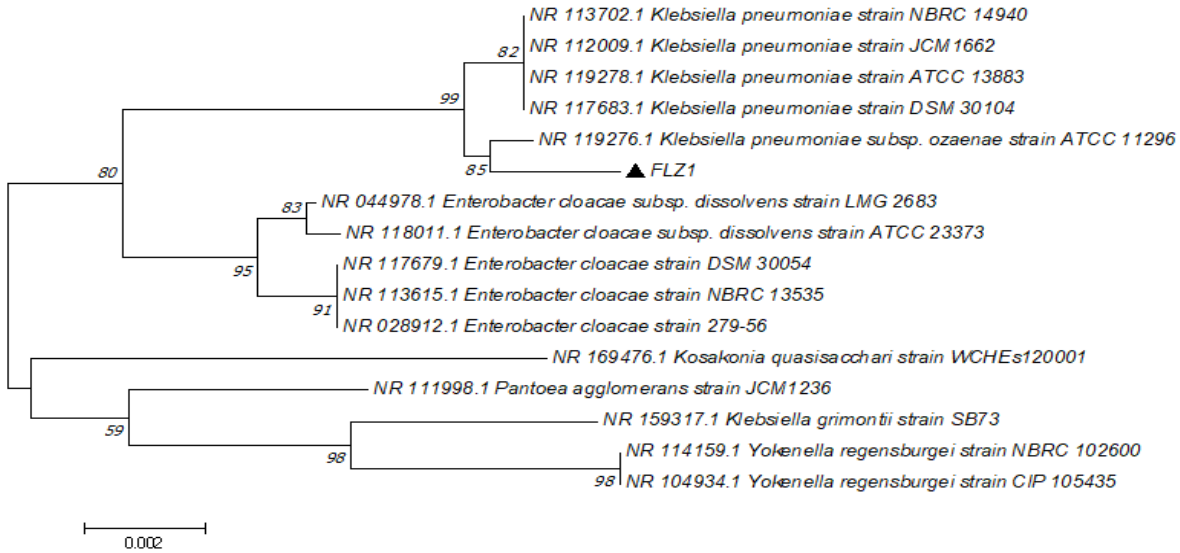

Figure 2. Phylogenetic analysis results of *Klebsiella pneumoniae* isolate (FLZ1).

plates. Among them, the first type of colony was white and rounded exhibiting the shape and appearance of yogurt drops, milky-white in the middle and gray-transparent on the outside, which suggested that the pathogen was *Klebsiella pneumoniae* (Figure 1A) and was named as FLZ1. The second type of colony morphology was grayish-white, moist, sticky, and exhibited a clear hemolytic ring around the colonies (Figure 1B), which indicated the presence of *Pm* and was named as FLZ2. The third type of colony was grayish-white, slightly yellow, smooth, convex, small, having a dry appearance, and exhibiting observable reflection under light (Figure 1C), which implied the existence of *Streptococcus* and was named as FLZ3.

Phylogenetic analysis

The BLAST analysis showed that the amplified target gene sequence of the suspected FLZ1 isolates was 100% consistent with those of *Klebsiella pneumoniae*. The phylogenetic tree that was constructed based on target gene sequences demonstrated that FLZ1 had the

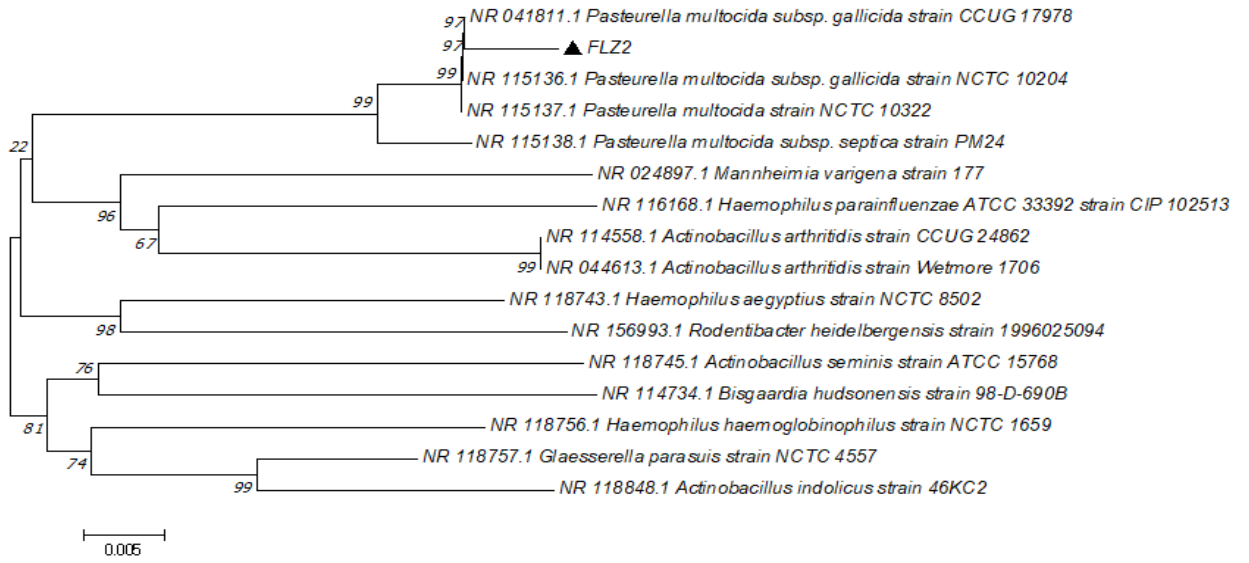

Figure 3. Phylogenetic analysis results of *Pasteurella multocida* isolate (FLZ2).

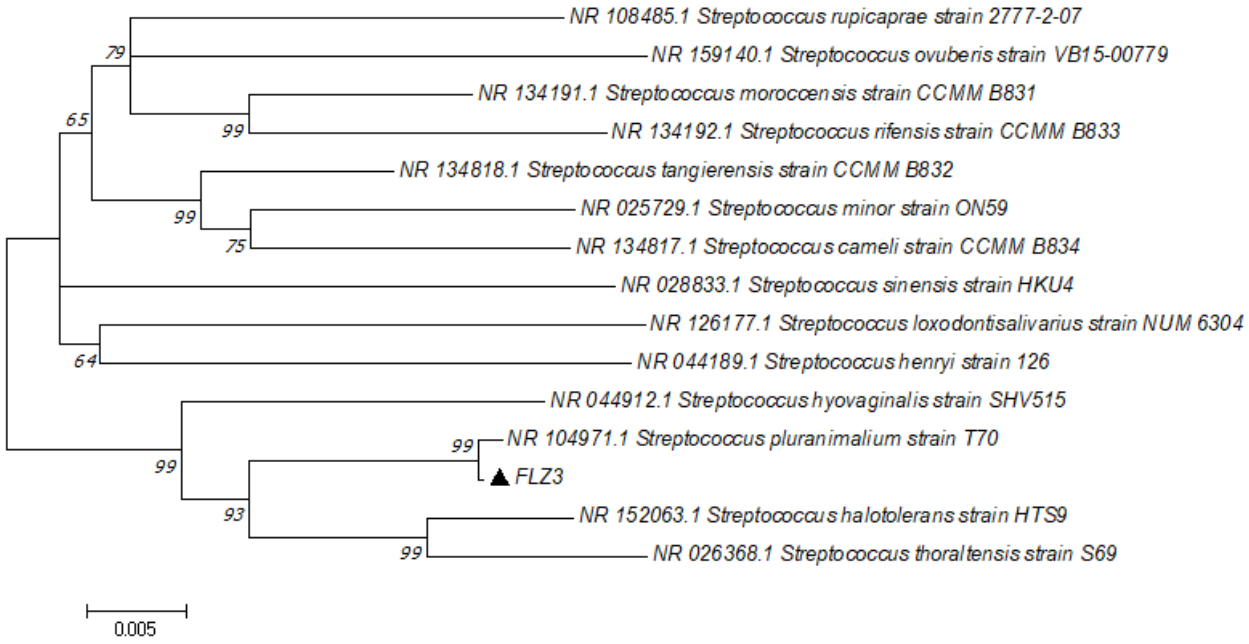

Figure 4. Phylogenetic analysis results of *Streptococcus pluranimalium* isolate (FLZ3).

highest homology with NBRC14940, JCM1662 ATCC13883, DSM30104, and other strains of *Klebsiella pneumoniae*, indicating close relation (Figure 2). The amplified target gene sequence of the suspected FLZ2 isolates was 100% consistent with that of *Pasteurella multocida*. According to the phylogenetic tree constructed based on the target gene sequences, the FLZ2 strains were in the same clade as the *Pm* strains, including CCUG17978 and NCTC10322, with up to 99% homology (Figure 3). The amplified target gene sequence of the suspected *Streptococcus*

pluranimalium (FLZ3) isolates was 100% consistent with the sequences of *Streptococcus pluranimalium*. The phylogenetic tree that was constructed based on the amplified target gene sequences showed that FLZ3 strains were in the same clade as T70 *Streptococcus zooniae* strain, with up to 99% homology (Figure 4).

Detection of antibiotic susceptibility

The antibiotic sensitivity tests of the three isolates were conducted by using 14 classes of 27 antibiotics (Table 1). The results showed that all three isolates were sensitive to amikacin, erythromycin, linezolid, enrofloxacin, and chloramphenicol. In addition, the *Klebsiella pneumoniae* strain (FLZ1) was sensitive to levofloxacin, piperacillin, ampicillin-sulbactam, ceftriaxone, cefazolin, ceftazidime, cefotaxime, aztreonam, and meropenem. The *Pasteurella multocida* strain (FLZ2) was sensitive to gentamicin, streptomycin, vancomycin, levofloxacin, minocycline, and tetracycline. The *Streptococcus pluranimalium* strain (FLZ3) was sensitive to gentamicin, streptomycin, vancomycin, ciprofloxacin, piperacillin, ampicillin, ampicillin-sulbactam, ceftriaxone, cefzolin, cefradine, cefuroxime, ceftazidime, cefotaxime, meropenone, minocycline, and tetracycline.

Discussion

In this study, the bacterial pathogens causing herd respiratory disease in dairy calves in the Ningxia region of China were isolated and cultured. Three bacterial species including *Klebber pneumoniae*, *Pasteurella multocida*, and *Streptococcus pluranimalium* were identified by both morphological and molecular investigations. The sensitivity of these pathogen isolates to 27 different antibiotics was also determined to provide a reference for the medicinal treatment of mixed infections involving these three bacterial species.

The sequencing and phylogenetic tree results showed that the isolated strain FLZ1 had high homology with *Klebsiella pneumoniae* which was known to cause pneumonia, diarrhea, meningitis, peritonitis, and septicemia in dairy cows. Meanwhile, this bacterium has been causing an increasing number of outbreaks in dairy cows in recent years, consequently causing serious harm to the aquaculture industry in China [13]. This bacterium has also been frequently detected in dairy farms in Ningxia, China in recent years, which caused respiratory symptoms in dairy calves and mastitis in lactating cows, and therefore, negatively impacted dairy cattle breeding in Ningxia, China.

The isolated bacterial strain FLZ2 was found to have high homology with *Pasteurella multocida*. This bacterium can cause hemorrhage and septicemia [14]. The identification of this pathogen explained the blackened appearance of blood observed in the current outbreak. The isolated bacterial strain FLZ3 was found to have high homology with *Streptococcus pluranimalium*. Currently, reports on cows infected with *Streptococcus pluranimalium* are rare. In ruminants, only Dong, *et al*. have reported in detail on goats infected with *Streptococcus pluranimalium*, which causes depression-like behavior [15]. Its symptoms in infected sheep included slight cough and panting, nasal mucosa, conjunctival flush, sticky secretions, and the inability to stand upright. In the current outbreak, calves had mainly exhibited respiratory symptoms with some infected cows also having nasal secretions, which was consistent with the symptoms observed in goats affected by *Streptococcus pluranimalium*.

The results of antibiotic sensitivity tests revealed that the three pathogen isolates were sensitive to amikacin, erythromycin, linezolid, ennofloxacin, and chloramphenicol, and had different degrees of resistance to the remaining 22 tested antibiotics. *Klebsiella pneumoniae* developed resistance to gentamicin, streptomycin, vancomycin, lincomycin, clindamycin, tetracycline, and some other common clinical drugs. In this studied outbreak of respiratory diseases occurring in cows and

Table 1. Antimicrobial susceptibility results.

Antibiotic type	Antibiotic agent	Content (μg)	*Klebsiella pneumoniae*		*Pasteurella multocida*		*Streptococcus pluranimalium*	
			Diameter of bacteriostatic zone (mm)	Result	Diameter of bacteriostatic zone (mm)	Result	Diameter of bacteriostatic zone (mm)	Result
Aminoglycoside	Amikacin	30	25.33	S	18.33	S	31.00	S
	Gentamicin	10	11.67	R	22.33	S	29.33	S
	Streptomycin	10	9.67	R	22.00	S	24.00	S
Macrolides	Erythromycin	15	11.00	S	25.00	S	30.00	S
polypeptide	Vancomycin	30	0.00	R	17.67	S	23.33	S
Oxazolidinones	Linezolid	30	12.33	S	28.33	S	36.00	S
Fluoroquinolones	Levofloxacin	5	23.00	S	27.00	S	8.00	R
	Ciprofloxacin	5	26.33	I	24.33	I	31.33	S
	Enoxacin	5	26.33	S	24.00	S	31.00	S
Phenicols	Chloramphenicol	30	18.00	S	26.67	S	22.00	S
Sulfonamides	Sulfamethoxazole	25	0.00	R	0.00	R	31.67	I
Lincosamides	Lincomycin	15	0.00	R	21.67	I	15.33	R
	Clindamycin	2	0.00	R	21.67	I	22.67	I
Penicillins	Piperacillin	100	21.67	S	14.67	R	32.67	S
	Ampicillin	10	0.00	R	7.50	R	30.00	S
β-lactam	Ampicillin-sulbactam	20	20.00	S	14.33	I	33.00	S
Cephalosporin	Ceftriaxone	30	34.00	S	11.00	R	32.00	S
	Cefazolin	30	26.67	S	11.67	R	33.00	S
	Cefradine	30	19.33	R	16.67	R	40.67	S
	Cefuroxime sodium	30	27.67	I	0	R	33.67	S
	Ceftazidime	30	31.67	S	11.33	R	26.00	S
	Ceftizoxime	30	34.00	S	12.33	R	35.00	S
Monobactams	Aztreonam	30	34.67	S	0.00	R	18.00	R
carbapenems	Meropenem	10	29.67	S	15.33	R	33.00	S
Tetracyclines	Tigecycline	15	23.33	I	22.67	I	27.33	I
	Minocycline	30	22.33	I	25.33	S	34.00	S
	Tetracycline	30	16.33	R	26.33	S	33.33	S

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

calves in Ningxia, China, resident veterinarians had employed gentamicin, tetracycline, and other antibiotics to treat the affected calves. However, the poor treatment effects were observed, which might attribute to the mixed infections that characterized this studied outbreak, in addition to the different degrees of drug resistance carried by pathogens throughout this area. Moreover, these antibiotics are also commonly used across many aquaculture farms. The results obtained by this study, like many others, once again indicated that the use of the same antibiotic for an extended period would inevitably lead to the generation of bacterial resistance. Therefore, timely drug rotation is recommended to address the issue of drug resistance in such outbreaks.

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