RESEARCH ARTICLE

Identification and histopathological analysis of *Streptococcus pluranimalium* caused pneumonia in sheep

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Sheep pneumonia is a common infectious disease of sheep respiratory system, which has a high incidence in lambs with weak immune systems. *Streptococcus pluranimalium* can be transmitted through the respiratory and digestive tracts of a variety of animals, which may cause infections in multiple tissues and organs, but rarely reported on sheep. The purpose of this study was to provide a scientific basis for the prevention and control of *Streptococcus pluranimalium* sheep pneumonia. The lung samples of a Dorper sheep were collected. The *Streptococcus pluranimalium* 16S rDNA sequences were amplified and sequencing confirmed. In addition, the histological changes were observed, and antimicrobial susceptibility analysis was performed. The results of colony morphology showed scattered, light gray, transparent, and small needle-like colonies with hemolysis around them, which indicated Gram-positive colonies and was chain-shaped. The phylogenetic tree showed that the genetic distances between the isolated strain and *Streptococcus pluranimalium* Th11417, 6a4R-CH01, B700bind 18-5 strains registered in GenBank were the closest. Considering the colony morphology, Gram staining microscopic examination, and 16s rDNA sequencing results, the isolated strains were identified as *S. pluranimalium* (Sp-1). *S. pluranimalium* can make the alveolar structure of the infected sheep unclear by causing a large amount of inflammatory cell infiltration in the alveolar cavity with obvious hyperemia. The isolated strain Sp-1 was sensitive to Amikacin, Gentamicin, and other nine antimicrobials, and was intermediate sensitive to Streptomycin and Aztreonam, while was resistant to Linezolid, Levofloxacin, and other sixteen antimicrobials. This study is the first report of *S. pluranimalium* pneumonia identified from a Dorper sheep in Ningxia Hui Autonomous Region, China. The results of this study provided a scientific basis for selecting effective antibiotics to treat *S. pluranimalium* pneumonia in sheep.

Keywords: sheep; *Streptococcus pluranimalium*; pneumonia; antimicrobials.

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Introduction

Sheep pneumonia is a common infectious disease of sheep's respiratory system, which has a high incidence in lambs with weak immune systems. The most common clinical signs of sheep pneumonia include elevated body temperature, mental tiredness, loss of appetite, cough, runny nose, dyspnea, and other clinical signs [1]. The main causes of pneumonia include

bacterial infection, low immunity, transport stress, environmental stress, and other factors [2]. The infection caused by *Pasteurella multocida* [3], *Mycoplasma pneumoniae* [4, 5], *Staphylococcus aureus* [6, 7], *Klebsiella pneumoniae* [8], and *Streptococcus pneumoniae* [9] has been reported.

Streptococcus pneumoniae is a Gram-positive bacterium with more than 90 serotypes, which is usually colonized in the upper respiratory tract, digestive tract, and urinary tract of animals [10]. *Streptococcus pluranimalium* (*S. pluranimalium*) was first isolated and named by Devriese, *et al*. [11], which can be transmitted through the respiratory tract and digestive tract [12] and causes infections in a variety of animals and affects multiple tissues and organs [12, 13]. It has been reported that *S. pluranimalium* was isolated from the reproductive tract of cattle [14, 15], milk samples [16], vaginal secretions of alpacas [17], meningoventriculitis with septicemic of a neonatal calf [18], nose swabs of sheep, lungs, kidneys, and subcutaneous edema fluid of pigs [13], respiratory tract of dogs [12], and meningoencephalitis in a horse [19], causing miscarriage [14, 15], meningitis [18], and mastitis in cattle and sheep [20, 21], meningitis and septicemia in calves [18], and endocarditis in adult chickens [22]. *S. pluranimalium* not only infected livestock in large-scale farms, but also infected wild animals. It was reported that *S. pluranimalium* isolated from *Pseudois nayaur* could cause weakness of limbs, difficulty in movement, shortness of breath, and other signs [23]. In recent years, more and more cases of *S. pluranimalium* infection in humans have been found to be associated with human infectious endocarditis, meningitis, and arthritis [16, 24, 25]. It can be transmitted to human through skin, mucosa, respiratory tract, and digestive tract. According to published reports, the bacterium has been isolated from patients with septicemia, meningitis, and endocarditis, while both infant and adult infections have been reported [10, 24, 26-28]. *S. pluranimalium* has been proved to cause a certain degree of harm to human health and animal industry. However, there is no report of *S. pluranimalium* infection on animals in Ningxia Hui Autonomous Region, China.

In August 2021, a Dorper sheep farm in Wuzhong City, Ningxia Hui Autonomous Region, bought a batch of Dorper sheep from another city without isolation observation. Three days later, some sheep showed the signs of pneumonia and died. In order to determine the cause of the disease, the pathological samples from a dead sheep were collected for pathogen isolation and identification. The analysis of 16S rDNA gene sequence, observation of histological changes, and determination of antimicrobial susceptibility were also performed, which will provide a scientific basis for the prevention and control of this disease in future.

Materials and methods

Sample collection

The lung tissue samples were collected from a 6-month-old female sheep with 13 days of history of pneumonia being diagnosed by a certified veterinary doctor. The sheep suffered from mental depression, cough, dyspnea, high fever, and died due to ineffective treatment. The lung tissues were removed and subjected to necropsy examination. The collected lung tissue samples were put into sterile sample tubes, and then, were transported to the Clinical Veterinary Laboratory at School of Agriculture, Ningxia University, Yinchuan, Ningxia, China within 2 h after collection for etiological diagnosis and histopathological observation.

Histological sample preparation and observation

The collected lung tissues were immersed in 10% neutral formaldehyde solution and fixed for 7 days. The fixed tissues were dehydrated by using automatic dehydrator (Junjie, Wuhan, Hubei, China) through the following steps: 75% ethanol for 4 h, 85% ethanol for 2 h, 95% ethanol for 1 h, 100% ethanol for 2 h with the fresh reagent change every 0.5 h, xylene for 20 min with the

reagent change every 10 min, paraffin wax for 6 h with the reagent change every 1 and 2 hours, respectively. The tissues were then embedded by using the embedding machine (Zhongwei, Changzhou, Jiangsu, China). The Leica-2016 rotary microtome (Leica Microsystems, Wetzlar, Germany) was used for sectioning and the automatic staining machine (Paisijie, Changzhou, Jiangsu, China) was employed for staining of tissues section through the following sequence: dewaxing, hematoxylin staining for 10-20 min, rinsing with water for 1-3 min, hydrochloric acid alcohol differentiation for 5-10 s, rinsing with water for 1-3 min, immersing in 50℃ water or weakly alkaline aqueous solution until blue color showing, rinsing with water for 1-3 min, adding 85% ethanol for 3-5 min, eosin staining for 3-5 min, washing with water for 3-5 s, gradient ethanol dehydration, xylene transparent, and neutral gum sealing. A 3DHISTECH digital slice scanner (Budapest, Hungary) was used to collect images on the slices.

Mycological culture

The collected lung tissue was sterilized with flame. Then, a small piece of deep lung tissue was cut out and inoculated on 5% defibrinated sheep blood agar plates (Guangzhou Hongquan Biotechnology Co., LTD, Guangzhou, Guangdong, China) under aseptic conditions. Each sample was repeated 3 times with Nutrient broth solid media (Qingdao hope bio-technology Co., LTD, Qingdao, Shandong, China), and was cultured at 37℃ for 24 h. The single colony was selected and inoculated on a new 5% defibrillated sheep blood agar plate and Nutrient broth medium under aseptic operation. The colony morphology was observed, and the single colony was selected for Gram staining and microscopic examination. The liquid culture was incubated in BSD-YX3200 constant temperature incubator (BoXun, Shanghai, China) at 37℃, 180 rpm for 24 hours.

DNA extraction and polymerase chain reaction

2 mL of bacterial culture was centrifuged at 12,000 rpm for 2 min to obtain the precipitation of the strain. The bacterial genomic DNA was extracted by using bacterial genomic DNA extraction kit (Tiangen, Beijing, China). The *Streptococcus pluranimalium* 16S rDNA forward primer (5'-GAATTCCGAGAGTTTGATCCTGGCT-3') and reverse primer (5'-AAGCTTGAGGTAATCCAT CCCCACGTTC-3') were employed according to the genome sequence in GenBank (access number: NZ_CP025536.1). The polymerase chain reaction (PCR) was performed by using PCR kit from TaKaRa (Osaka, Japan) with the total volume of 25 μL including 2.5 μL of 10× PCR buffer, 2.0 μL of dNTP mixture, 0.125 μL of rTaq, 1 μL of primer, 0.5 μL of DNA, and ddH_2O up to 25 μL. The reaction condition was 95℃ for 2 min followed by 30 cycles of 95℃ for 30 s, 53℃ for 20 s, and 72℃ for 2 min, then 72℃ for 8 mins. *Streptococcus pneumoniae* ATCC 49619 was used as a positive control. The PCR products were electrophoresis analyzed with 1% agarose gel and then were purified by using E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Norcross, Georgia, USA). The purified products were sent to Shenggong Bioengineering Co., Ltd (Shanghai, China) for sequencing.

Phylogenetic analysis

The sequencing data of the isolated strains were spliced by using LaserGene software (DNAstar, Madison, Wisconsin, USA), and the spliced sequences were compared to each other by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). MegAlign (DNAstar, Madison, Wisconsin, USA) was employed to analyze the identity of isolate sequences. The phylogenetic tree of the isolated strains was constructed based on the sequences of 16S rDNA by using the adjacency Neighbor-joining (NJ) method in MEGA7.0 software (Madison, Wisconsin, USA), and the genetic evolution was then analyzed.

Antimicrobial susceptibility testing

According to Performance for Antimicrobial Susceptibility Testing from the 28th Edition of Clinical and Laboratory Standards Institute (https://clsi.org), Kirby-Bauer method was applied for this study. Briefly, the colony suspension was equivalented to a 0.5 McFarland standard. The colony suspension was then evenly coated on Mueller-Hinton agar (MHA) with 5%

sheep blood under aseptic operation. After the surface of the medium was dried, the antimicrobial sensitive paper was pasted on the surface of the culture medium. The diameter of the bacteriostatic zone was measured after being incubated in a constant temperature incubator at 37℃ for 18-20 hours. *S. pneumoniae* ATCC 49619 was used as the quality control strain. Each antimicrobial was repeated three times. The criteria for the interpretation of zone diameter used in this study were described in Table 1.

Results

Histopathological observation

The necropsy examination found that the lungs showed tissue swelling, surface congestion, and bleeding (Figure 1). Histological observation found that the alveolar structure was unclear with obvious hyperemia, red blood cell aggregation, and bronchial epithelium exfoliation. There was a large amount of inflammatory cell infiltration in the alveolar cavity with obvious hyperemia (Figure 2).

Figure 1. Histopathological changes in the lung.

Colony morphology

After the tissue samples inoculated in the solid medium of defibered sheep blood were cultured at 37℃ for 24 hours, scattered light gray, transparent, small needle-like colonies, and hemolysis around the colonies were observed. Gram staining and microscopic examination showed that the strain was Gram-positive and chain-shaped, which was named SP-1.

PCR identification and phylogenetic analysis

The 16S rDNA PCR amplification products of isolates were analyzed by 1% agarose gel electrophoresis. The results showed that the length of the amplified fragments was about 1,500 bp. The amplification sequences were analyzed by the Sequence distances method of MegAlign software. The results showed that the isolated strain Sp-1 had 99.7% identity to *S. pluranimalium* strain Th11417 (Figure 3). The phylogenetic tree demonstrated that Sp-1 was on the same branch as *S. pluranimalium* Th11417 and it was distantly related to *Streptococcus halotolerans*, *Streptococcus hyovaginalis*, *Streptococcus rupicaprae*, *Streptococcus thermophilus*, *Streptococcus parasuis*, and *Streptococcus sinensis* (Figure 4).

Antimicrobial susceptibility analysis

The antimicrobial susceptibility analysis of *S. pluranimalium* to 27 antibiotics were shown in Table 1. The isolated strain Sp-1 was sensitive to Amikacin, Gentamicin, Erythromycin, Meropenem, Ampicillin, Sulbactam, Ceftazidime, Tigecycline, Minocycline, and was intermediate sensitive to Streptomycin and Aztreonam, while was resistant to Linezolid, Levofloxacin, Ciprofloxacin, Enrofloxacin, Norfloxacin, Sulfamethoxazole, Lincomycin, Clindamycin, Piperacillin, Ampicillin, Ceftriaxone, Cefazolin, Cefuroxime, Cefotaxime, Cefradine, and Tetracycline.

Discussion

S. pluranimalium was first discovered by Devriese, *et al*., which was isolated from subclinical mastitis, the genital tract and tonsil of cattle, tonsil of goat and cat, the respiratory tract of canaries [11]. It was also reported that *S. pluranimalium* was isolated from goat nasal swabs in Jilin Province, China [29]. *S.*

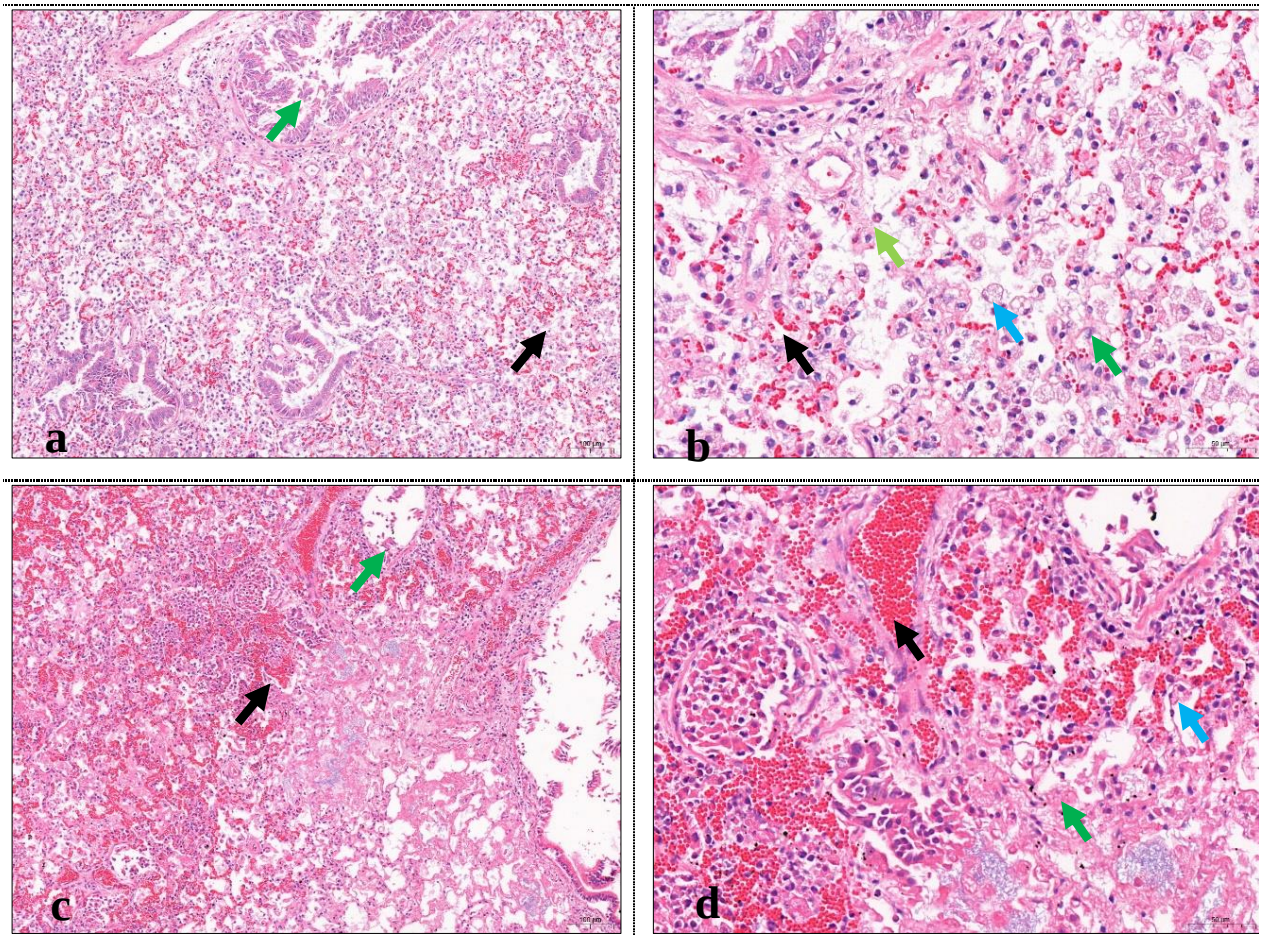

Figure 2. Histological observation. **a.** Lung bronchial epithelium shedding (↑) and hyperemia (↑) (HE, ×100). **b.** Pulmonary alveolar epithelial cell lysis (↑), neutrophil exudation in the alveolar cavity (↑), foam cell (↑), hyperemia (↑) (HE, ×400). **c.** Bronchial epithelium in the alveolar cavity (↑) and hyperemia (↑) (HE, ×100). **d.** Alveolar epithelial cell lysis (↑), foam cell (↑), hyperemia (↑) (HE, ×400).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12		
1		99.7	99.0	98.9	98.4	98.4	98.3	96.9	96.1	96.1	95.9	96.0	1	Sp-1
2	0.3		99.1	99.1	98.6	98.5	98.4	97.0	96.3	96.3	96.1	96.2	2	S.pluranimalium_TH11417
3	1.1	0.9		98.9	98.1	97.9	97.8	96.4	95.9	95.9	95.6	95.6	3	Streptococcus_sp.6a4R-CH01
4	1.2	0.9	1.2		97.9	97.8	97.9	96.2	95.9	95.9	95.4	95.3	4	Streptococcus_sp.B700-18-5
5	1.6	1.4	1.9	2.1		98.7	98.6	96.5	96.0	96.0	96.2	96.3	5	S.halotolerans_HTS9
6	1.6	1.5	2.1	2.2	1.3		99.9	96.6	95.6	95.6	96.3	96.1	6	S.hyovaginalis_TRG26
7	1.7	1.6	2.2	2.1	1.4	0.1		96.5	95.6	95.6	96.2	96.0	7	S.hyovaginalis_SHV515
8	3.2	3.1	3.7	3.9	3.6	3.5	3.6		95.6	95.6	96.7	96.1	8	S.rupicaprae_2777-2-07
9	4.0	3.8	4.2	4.2	4.1	4.5	4.5	4.5		100.0	97.3	97.2	9	S.thermophilus_4615
10	4.0	3.8	4.2	4.2	4.1	4.5	4.5	4.5	0.0		97.3	97.2	10	S.thermophilus_TW12-4
11	4.2	4.0	4.5	4.7	3.9	3.8	3.9	3.4	2.7	2.7		98.4	11	S.parasuis_SUT483
12	4.1	3.9	4.5	4.8	3.8	4.0	4.1	4.0	2.8	2.8	1.6		12	S.sinensis_11026353
	1	2	3	4	5	6	7	8	9	10	11	12		

Figure 3. The identity of Sp-1 with *Streptococcus spp*.

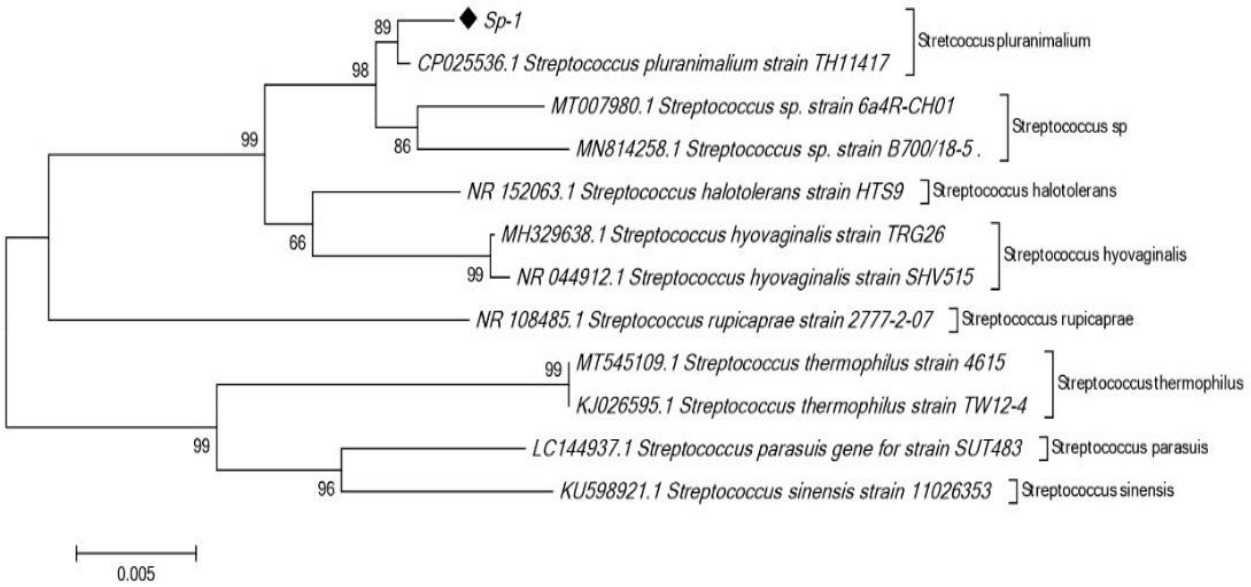

Figure 4. Phylogenetic tree of isolated strains constructed based on 16S rDNA gene sequence.

Table 1. Antimicrobial susceptibility testing.

Antibiotic family	Antibiotics	Disk content	Zone Diameter Breakpoints			Strain (SP-1)	Susceptibility
			S	I	R		
Aminoglycosides	Amikacin	30	≥17	15-16	≤14	25.67±0.47	S
Aminoglycosides	Gentamicin	10	≥15	13-14	≤12	23.00±0.47	S
Aminoglycosides	Streptomycin	10	≥15	12-14	≤11	14.00±0.00	I
Macrolides	Erythromycin	15	≥21	16-20	≤15	22.00±0.00	S
Glycopeptides	Vancomycin	30	≥17	-	-	13.00±0.00	R
Oxazolidinones	Linezolid	30	≥21	-	-	0.00±0.00	R
Fluoroquinolones	Levofloxacin	5	≥17	14-16	≤13	13.00±0.00	R
Fluoroquinolones	Ciprofloxacin	5	≥21	16-20	≤15	9.00±0.00	R
Fluoroquinolones	Enrofloxacin	5	≥21	16-20	≤15	9.00±0.00	R
Fluoroquinolones	Norfloxacin	10	≥17	13-16	≤12	0.00±0.00	R
Sulfonamides	Sulfamethoxazole	25	≥16	11-15	≤10	0.00±0.00	R
Lincosamides	Lincomycin	15	-	-	-	0.00±0.00	R
Lincosamides	Clindamycin	2	≥19	16-18	≤15	13.33±0.47	R
Carbapenems	Meropenem	10	-	-	-	31.33±.0.47	S
β-lactams	Piperacillin	100	≥21	18-20	≤17	10.00±0.82	R
β-lactams	Ampicillin	10	≥26	19-25	≤18	0.00±0.00	R
β-lactams	Ampicillin and Sulbactam	20	≥20	-	-	24.33±0.47	S
β-lactams	Ceftriaxone	30	≥27	26	≤25	11.33±047	R
β-lactams	Cefazolin	30	≥18	15-17	≤14	0.00±0.00	R
β-lactams	Cefuroxime	30	≥23	15-22	≤14	0.00±0.00	R
β-lactams	Ceftazidime	30	≥18	15-17	≤14	22.00±0.82	S
β-lactams	Cefotaxime	30	≥28	26-27	≤25	13.00±0.00	R
β-lactams	Cefradine·	30	≥18	15-17	≤14	11.67±0.47	R
β-lactams	Aztreonam	30	≥22	16-21	≤15	17.33±0.47	I
Tetracyclines	Tigecycline	15	≥19	18	≤17	28.00±0.00	S
Tetracyclines	Minocycline	30	≥23	19-22	≤18	24.00±0.00	S
Tetracyclines	Tetracycline	30	≥23	19-22	≤18	0.00±0.00	R

S: susceptible; I: intermediate; R: resistant; -: no relevant data.

pluranimalium was found not only in animal breeding farms, but also in wild animals. *S. pluranimalium* has been reported to be isolated from the lung of a wild animal *Pseudois nayaur* in Qinghai Province, China [23]. This study is the first report that *S. pluranimalium* was isolated and identified from a case of pneumonia of a Dorper sheep in Ningxia Hui Autonomous Region, China. There are rarely reports that *S. pluranimalium* can cause pneumonia in sheep. Although *S. pluranimalium* was discovered more than 20 years ago, there are only a few studies on it. The pathogenic mechanism is still not clear, and the existing corresponding diagnostic techniques have some degree of limitations. Therefore, it is necessary to study the isolation method of *S. pluranimalium*, the pathological changes in lung lesions, and the sensitivity to multiple antibiotics, to lay the foundation for the prevention and control and the pathogenesis research of *S. pluranimalium*.

In this study, a colony was isolated from the lungs of sheep who died of clinical pneumonia. The morphology of the colony was light gray, transparent, and small needle shaped. The colony was surrounded by hemolysis. The results of Gram staining showed that it was Gram-positive bacteria and a chain-like arrangement of pathogenic bacteria, which was consistent with the colony morphology reported by Devriese, *et al*. [11]. The results of phylogenetic tree constructed according to 16s rDNA sequence showed that the genetic distances between the isolated strain and *Streptococcus pluranimalium* Th11417, 6a4R-CH01, B700bind 18-5 strains registered on GenBank were the closest. Considering the colony morphology, Gram staining microscopic examination results, and 16s rDNA sequencing results of the isolated strains, the isolated strains were identified as *S. pluranimalium* (Sp-1).

Through the histopathological observation of the lung of diseased sheep, the alveolar structure of the diseased sheep was severely damaged with obvious hyperemia, exfoliation of bronchial epithelium, and a large amount of inflammatory cell infiltration in the alveolar cavity. In previous study, *S. pluranimalium* isolated from the respiratory tract of dog was injected into the abdominal cavity of BALB/c mice. After 96 hours of injection, the pathological sections of the lungs showed the increase of inflammatory cells, while the alveolar epithelial cells dissolved, and there were serious severe symptoms such as pulmonary hemorrhage [12], which is consistent with the findings of this study.

The results of antimicrobial susceptibility testing showed that the isolated strain Sp-1 was sensitive to Amikacin, Gentamicin, Erythromycin, Meropenem, Ampicillin, Sulbactam, Ceftazidime, Tigecycline, and Minocycline; intermediate sensitive to Streptomycin and Aztreonam; and resistant to Linezolid, Levofloxacin, Ciprofloxacin, Enrofloxacin, Norfloxacin, Sulfamethoxazole, Lincomycin, Clindamycin, Piperacillin, Ampicillin, Ceftriaxone, Cefazolin, Cefuroxime, Cefotaxime, Cefradine, and Tetracycline. Pan, *et al*. analyzed the whole genome sequence of *Streptococcus pluranimalium* and found that the strain contained three resistance genes, *mef* (A), *msr* (D), and *lnu* (C), which led to the resistance of the strain to Erythromycin and Lincomycin [30]. In this study, the isolated strain Sp-1 showed resistance to Lincomycin. In the study of Zhang, *et al*., the isolated strain was sensitive to Erythromycin [23], which was consistent with the results of this experimental study. In the Antimicrobial susceptibility testing of Wang, *et al*. the strains isolated was resistant to Norfloxacin, Sulfamethoxazole, Tobramycin, Gentamicin, and sensitive to Amoxicillin, Ceftriaxone sodium, and Mezlocillin [13], which were similar to the results of this study.

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