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Original Article

The epidemiology of drug resistance in Streptococcus species isolated from yaks in Tibet

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Abstract

This study aimed to investigate the antibiotic sensitivity and resistance profiles of *Streptococcus* isolated from diarrhea in yaks in Tibetan regions. We successfully isolated and identified *Streptococcus* from fecal samples using morphological analysis and PCR amplification. Subsequent bacterial susceptibility tests and resistance gene detection revealed that the overall isolate rate of *Streptococcus* from yaks in Tibet was 57.68%, with Changdu recording the highest (71.43%) and Naqu the lowest (34.09%). The bacteria exhibited significant resistance to macrolides, particularly Erythromycin (52.86%) and Midecamycin (52.14%), β-Lactam resistance, including Ceftazidime, Cephazolin, and Carbenicillin, was the lowest at 7.14%, 2.15%, and 7.14%, respectively. Among the 140 isolates, 37 distinct resistance patterns were identified, with ERY/MID being the most prevalent for Macrolides. The detection rates for the resistance genes were as follows: *erma* (38.36%), *ermb* (65.20%), *mefa* (28.93%) for Macrolides; *tetk* (13.42%), *tetl* (14.47%), *teto* (11.53%), and *tetm* (20.34%) for Tetracyclines; and *pbp2b* (5.24%) for β-lactams. The conformity between resistance phenotypes and genes was high, with 82.83% for Tetracyclines, 89.14% for Macrolides, and 95.80% for β-lactams. These findings indicate that *Streptococcus* in yaks exhibits a predominant resistance to macrolides, accompanied by multiple resistance patterns, with the ERY/MID pattern dominant.

Keywords: Yaks, *Streptococcus,* Isolation and identification, Susceptibility test, Resistance analysis

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Introduction

Streptococcus, a genus of Gram-positive cocci often arranged in pairs or chains, is widely distributed in nature (Tian et al., 2019). These bacteria are common pathogens, including both pathogenic and non-pathogenic strains. Among those associated with cattle, species such as *Streptococcus agalactiae*, *Streptococcus bovis*, *Streptococcus mastitis*, and *Streptococcus pneumoniae* are prevalent, causing diseases such as mastitis, endocarditis, metritis, and pneumonia in cattle (Tevdorashvili et al., 2015). Newborn calves under 3 weeks of age are particularly susceptible to *Streptococcus pneumoniae* infections, posing a significant threat to their health.

In 2015, data from China Antimicrobial Resistance Surveillance System bacterial resistance surveillance showed that most of the A, B, C, and G groups of β-hemolytic Streptococci, including *Streptococcus agalactiae*, remained highly sensitive to Penicillin, with resistance rates below 7% (Dongjing et al., 2024). However, the resistance rate for green-stained *Streptococcus*, isolated from sterile body fluids such as blood, was 9.0% against Penicillin. Strains of *Streptococcus pneumoniae* sensitive to Penicillin had high resistance rates to Erythromycin, Clindamycin, Aminomycin, Tetracycline, SMZ-TMP, and others, but remained relatively susceptible to Penicillins and Cephalosporins (Chen et al., 2023; Wu et al., 2024). Penicillin-resistant strains (PISP+PRSP) showed high resistance to Erythromycin and Clindamycin, up to 90-100%, some strains developing resistance to certain β-lactams like Ceftazidime and Ceftriaxone, but remained sensitive to vancomycin and linezolid, as well as Carbapenems (Zhou et al., 2018). A 2017-2018 study revealed that Vancomycin and Levofloxacin did not have resistant strains, while other antibiotics had varying resistance rates. Erythromycin and Clindamycin had resistance rates greater than 90%, while drugs such as Rifampicin, Cefepime, Ceftiofur, Meropenem, Chloramphenicol and Tetracycline had higher sensitivity rates. Approximately 87.84% of the isolates were resistant to multidrug (Zhang et al., 2018). The French surveillance network for antimicrobial resistance in pathogenic bacteria of animal source, a long-term monitoring initiative in Europe, showed that resistance rates for Erythromycin and Clindamycin decreased from 24% in 2006-2007 to 17% and remained stable until 2013, before rising again to around 22%. Tetracycline resistance gradually increased (from 14% in 2006 to 21% in 2015), while Streptomycin had the lowest resistance rate (11-16% between 2006 and 2015), although it was among the highest for Aminoglycoside resistance (Haenni et al., 2018; Bourély et al., 2020).

The resistance patterns of the *Streptococcus species* are complex and dynamic, necessitating the selection and use of antibiotics based on local epidemiology and resistance data. However, there is a lack of information on *Streptococcus* antibiotic resistance in Tibetan yaks. Therefore, this study collected 827 fecal samples from yak farms in Tibet between June 2020 and June 2022 to identify and analyze resistance of *Streptococcus* strains isolated from diarrhea cases. The study aimed to provide information on the control of *Streptococcus* infections in Tibet and inform future clinical practices.

Material and Methods

Sample collection and bacterial isolation and purification

A total of 827 fresh bovine diarrhea fecal samples were collected from seven different regions in the Tibet Autonomous Region, including Lasa, Naqu, Linzhi, Rikaze, Changdu, Ali and Shannan, from June 2020 to June 2022. Specific sampling details are provided in Table 1.

Table-1. Sample Distributions

Samples collection site	Lasa	Linzhi	Naqu	Rikaze	Changdu	Ali	Shannan
Elevation/meter	3650	2954	4510	3940	3257	4280	3572
Sample sizes	219	121	88	112	42	55	190

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The bovine diarrhea fecal samples were inoculated on TSB agar and incubated at 37 ° C for 12-18 hours. The bacterial suspension was streaked onto Columbia agar with 5% sterile defibrinated sheep blood using the three-tube method. The agar was incubated at 37 ° C for another 12-18 hours. Individual suspected cocci colonies were harvested and further cultured in TSB at 37 ° C for 12-18 hours, followed by Gram staining for morphological observation under a microscope.

PCR Identification

A set of universal 16S rRNA primers and *Streptococcus*-specific primer, designed according to the method of Good MF et al., 2020, were synthesized by BGI (Shanghai) Biological Engineering Co., Ltd. (primer information provided in Table 2). The PCR reaction mixture (25 μL) consisted of Premix Taq (TaKaRa Taq Version 2.0 plus dye) 12.5 μL, 10 μM of each primer upstream and downstream, 2 μL of DNA template and 8.5 μL of ddH2O. The PCR conditions were as follows: initial denaturation at 95 ° C for 5 minutes; denaturation at 95 ° C for 30 seconds; annealing at 50 ° C for 30 seconds; extension at 72 ° C for 40 seconds, for a total of 35 cycles; and final extension at 72 ° C for 10 minutes. 10 μL of the PCR product was loaded onto a 0.1% agarose gel for electrophoresis and the results were photographed and recorded. The PCR products were sent to the BGI sequencing department in Chengdu for sequencing.

Table-2. 16S rRNA universal primer and *Streptococcus*-specific primer sequences

Primers	Primer Sequences	Product Size/bp
16S rRNA	F:AGAGTTTGATCCTGGCTCAG	1500
	R:TACCTTGTTACGACTT	
Streptococcus-specific primer	F:GATACATAGCCGACCTGAGA	561
	R:AGGGCCTAACACCTAGCACT	

Antibiotic susceptibility testing

The antibiotic susceptibility test was performed using the Kirby-Bauer disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI) (Pompilio A et al., 2019). A total of 20 isolates from each of the seven Tibetan regions were tested. 500 μL of the bacterial suspension was evenly spread on MH agar plates to perform the test. Eleven antibiotic discs were placed in the center of each plate and incubated at 37 ℃ in an inverted position for 24 hours (These antimicrobial susceptibility tablets were purchased from Hangzhou Microbial Reagent Co. Ltd). The diameter of the inhibition zones was measured using a caliper. The results were interpreted according to the CLSI guidelines for executing antimicrobial susceptibility tests (Yi et al., 2020) and the manufacturer's instructions. The resistance determination criteria are provided in Table 3.

Table-3. Criteria for determining the results of antimicrobial susceptibility test.

Type of Antibiotics	Concentration (μg)	Diameter of the bacteriostatic zone/mm		
		Resistance	Intermediate	Sensitive
Tetracycline	30.00	≤18	19～22	≥23
Doxycycline	30.00	≤12	13～15	≥16
Minocycline	30.00	≤14	15～18	≥19
Erythromycin	15.00	≤15	16～20	≥21
Midecamycin	30.00	≤13	14～17	≥18
Ceftazidime	30.00	≤14	15～17	≥18
Cephazolin	30.00	≤14	15～17	≥18
Carbenicillin	10.00	≤13	14～16	≥17
Amikacin	30.00	≤14	15～16	≥17
Gentamicin	10.00	≤12	13～14	≥15
Neomycin	10.00	≤11	12～14	≥15
Kanamycin	30.00	≤13	14～17	≥18

Detection of drug resistance genes

Drug resistance gene primers refer to the reference (Waites KB et al., 2011). The specific primers are shown in Table 4. Beijing Dingfeng Biotechnology Co., LTD synthesized all of the primers. In this experiment, a 25 μL reaction system was used.

Table-4. Primer Sequences of Streptococcal Drug Resistance Genes

Gene Names	Primer sequence	Destination fragment/bp	Annealing temperature/℃
ermB	F：ATTGGAACAGGTAAAGGGC R：GAACATCTGTGGTATGGCG	442	50
ermA	F：TCTAAAAAGCATGTAAAAGA R：CTTCGATAGTTTATTAATATTAGT	645	52
mefA	F：AGTATCATTAATCACTAGTGC R：TTCTTCTGGTACTAAAAGTGG	346	53
tetM	F：GAACTCGAACAAGAGGAAAGC R：ATGGAAGCCCAGAAAGGAT	740	55
tetO	F：AACTTAGGCATTCTGGCTCAC R：TCCCACTGTTCCATATCGTCA	519	52
tetL	F：TGAACGTCTCATTACCTG R：ACGAAAGCCCACCTAAAA	993	50
tetK	F：TCCTGGAACCATGAGTGT R：AGATAATCCGCCCATAAC	189	50
pbp2b	F：GATCCTCTAAATGATTCTCAGGTGG R：CCATTAGCTTAGCAATAGGTGTTGG	1500	55

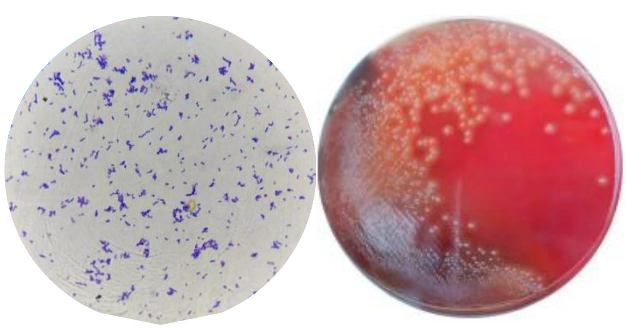
Results

Bacterial Isolation and Identification

A total of 477 streptococci were isolated from samples collected in seven cities in Tibet, with detailed isolation results presented in Table 5. In Gram staining, the bacteria appeared as blue-purple gram-positive cocci arranged in chains. Detailed results are shown in Figure 1.

Table-5. Isolation of samples from different cities in Tibet

Sample collection site	Lasa	Linzhi	Naqu	Rikaze	Changdu	Ali	Shannan
Sample size	117	78	30	68	30	32	122
Bacterial isolation rate/%	53.42	64.47	34.09	60.71	71.43	58.19	64.21



A B

Figure-1. Growth morphology and staining microscopy of *Streptococcus* in blood plates. A: Gram stain microscopy (× 1000) B: Sheep blood plating medium.

Results of 16S rRNA and streptococcal-specific primer PCR

The PCR products of the 16S rRNA primer for isolated strains showed a band at 1500 bp (Figure 2), consistent with the expected size. The PCR products of the streptococcal-specific primer for isolated strains showed a band at 561 bp (Figure 3), which also matches the expected result.

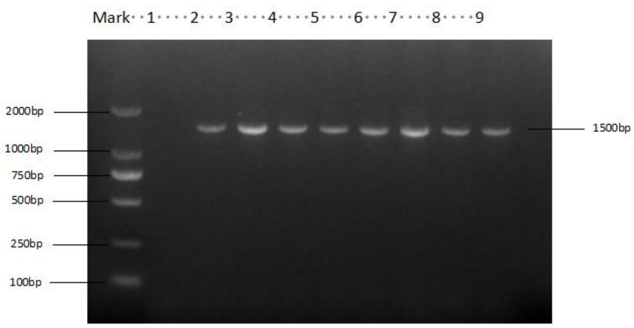


Figure-2. Gel electrophoresis results of 16S rRNA primers gel electrophoresis of some strains

Note: 1: Mark: DL 2000 DNA Marker ; Negative control; 2: Positive control

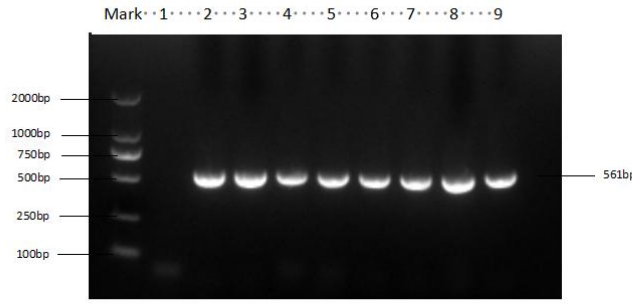


Figure-3. Gel electrophoresis results of PCR products of specific primers for some strains of *Streptococcus*

Note: 1: Mark: DL 2000 DNA Marker ; Negative control; 2: Positive control

Drug susceptibility testing

According to standard criteria, the resistance rates of yak streptococci from the seven Tibetan cities to macrolides (Erythromycin and Midecamycin) were high, at 52.86% and 52.14%, respectively. Tetracycline (Tetracycline, Doxycycline, and Minocycline) had the second highest resistance rate, at 25.00%, 18.57% and 14.29%, respectively. The lowest resistance was observed for Cephazolin, 2.15%. Detailed results are given in Table 6.

Table-6. Results of the antimicrobial resistance phenotype test of 140 strains derived from *Streptococcus* yak in seven cities of Tibet

Antibiotics group	Antibiotics	Sensitive		Intermediate		Resistance	
		No. strains	Percent (%)	No. strains	Percent (%)	No. strains	Percent (%)
Tetracyclines	Tetracycline	92	65.71	13	9.29	35	25.00
	Doxycycline	101	72.14	13	9.29	26	18.57
	Minocycline	94	67.14	26	18.57	20	14.29
Macrolides	Erythromycin	43	30.71	23	16.43	74	52.86
	Midecamycin	45	32.14	22	15.72	73	52.14
β-lactam	Ceftazidime	125	89.29	5	3.57	10	7.14
	Cephazolin	136	97.14	1	0.71	3	2.15
	Carbenicillin	127	90.72	3	2.14	10	7.14
Aminoglycosides	Amikacin	122	87.14	6	4.29	12	8.57
	Gentamicin	124	88.57	7	5.00	9	6.43
	Neomycin	118	84.29	15	10.71	7	5.00
	Kanamycin	113	80.72	15	10.71	12	8.57

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Analysis of drug resistance profiles

The drug resistance profiles of 140 yak streptococci to 12 common antibiotics are shown in Table 7. A total of 37 distinct resistance profiles were identified among the 140 strains, with abroad and scattered distribution. The resistance profile of ERY / MID was the most prevalent, as detailed in Table 7.

Table-7. Analysis of the drug resistance spectrum of 140 strains of *Streptococcus*

DRP	No. strains	DRP	No. strains	DRP	No. strains
ERY/GEN	1	DOX/ERY/MID	3	TCY/DOX/MNO/ERY/MID	4
ERY/MID	18	ERY/MID/AK	4	TCY/DOX/MNO/ERY/SRB	2
TCY/MNO	1	ERY/AK/KAN	1	TCY/DOX/ERY/MID/SRB	2
TCY/DOX	2	TCY/MID/GEN/KAN	1	TCY/DOX/ERY/MID/KAN	1
CAZ/AK	1	TCY/DOX/ERY/MID	4	DOX/ERY/MID/AK/GEN	1
CAZ/CAO	1	TCY/MNO/ERY/MID	2	MNO/ERY/MID/CAZ/SRB	2
MID/CAZ	1	TCY/DOX/MNO/MID	1	TCY/DOX/MNO/ERY/MID/SRB	3
MNO/ERY	1	TCY/ERY/MID/SRB	1	TCY/DOX/ERY/MID/AK/GEN	2
AK/KAN	1	DOX/MNO/ERY/MID	2	TCY/DOX/ERY/MID/CAZ/NEO	1
NEO/KAN	1	ERY/MID/CAZ/CZO	1	TCY/ERY/MID/CAZ/AK/NEO/KAN	1
TCY/ERY/MID	2	ERY/MID/CAZ/AK	1	TCY/ERY/MID/CAZ/SRB/GEN/NEO/KAN	1
TCY/ERY/KAN	1	ERY/MID/NEO/KAN	1	TCY/DOX/ERY/MID/CZO/SRB/GEN/NEO/KAN	1
ERY/MID/GEN	2				

Note: Drug Resistance Profile: DRP

Sensitivity to antibiotics in different Tibetan regions

As shown in Table 8, the seven cities of Tibet had the highest resistance to macrolides. Lasa, Naqu, Changdu, Ali, and Shannan had the highest sensitivity to beta-lactams, while Linzhi had the highest sensitivity to Tetracyclines, and Rikaze had the highest sensitivity to Aminoglycosides.

Table-8. Sensitivity analysis of *Streptococcus* to four types of antibiotics in different regions of Tibet.

	β-lactam			Tetracyclines			Macrolides			Aminoglycosides		
	S	I	R	S	I	R	S	I	R	S	I	R
Lasa	86.66	6.67	6.67	60	20	20	47.5	15	37.5	66.25	12.5	21.25
Linzhi	83.33	5	11.67	91.66	1.67	6.67	32.5	15	52.5	87.5	7.5	5
Naqu	90	0	10	70	6.67	23.33	27.5	22.5	50	85	7.5	7.5
Rikaze	93.33	0	6.67	63.33	8.33	28.34	27.5	15	57.5	100	0	0
Changdu	98.33	1.67	0	91.67	3.33	5	55	15	30	86.25	11.25	2.5
Ali	100	0	0	46.67	20	33.33	17.5	20	62.5	93.75	5	1.25
Shannan	95	1.67	3.33	55	26.67	18.33	12.5	10	77.5	77.5	10	12.5

Note: Units are %Sensitive: S; Intermediate: I; Resistance: R.

Heat map of antibiotic resistance

The heat map of antibiotic resistance for 140 yak streptococci from the seven Tibetan cities is presented in Figure 4, illustrating similarities and differences in resistance to the 12 antibiotics.

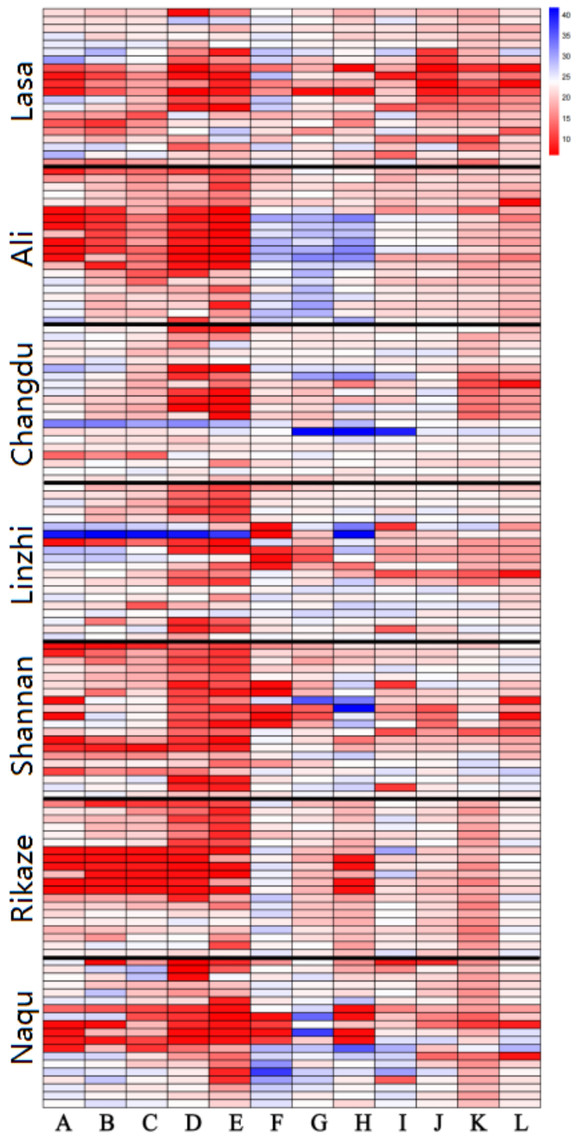

Figure-4. Heat map of antimicrobial resistance of 140 strains of yak-derived *Streptococcus* in seven cities in Tibet. The left ordinate represents different regions, and the abscissa represents different antibiotics (A-L). The color scale bar indicates the suppression range between 0~40mm. A:Tetracycline; B:Doxycycline, C:Minocycline, D:Erythromycin, E:Midecamycin, F:Ceftazidime, G:Cephazolin, H: Carbenicillin, I: Amikacin, J: Gentamicin, K: Neomycin, L: Kanamycin

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Detection of resistance genes

The PCR results for streptococcal resistance genes are shown in Table 9. The highest detection rate was for the ermB gene (65.20%), followed by ermA (38.36%), and the lowest rate was for the pbp2b gene (5.24%).

Table-9. Results of drug resistance gene detection of 477 yak-derived streptococcal isolates in seven cities of Tibet

Gene Name	Lasa	Linzhi	Naqu	Rikaze	Changdu	Ali	Shannan	Total rate (%)
tetK	13	7	7	14	0	11	12	13.42
tetL	26	0	8	17	3	8	7	14.47
tetO	8	0	4	3	0	4	36	11.53
tetM	32	12	2	28	2	12	9	20.34
ermA	13	47	9	27	3	16	68	38.36
ermB	69	43	23	39	12	22	103	65.20
mefA	39	1	11	32	8	0	47	28.93
pbp2b	7	8	2	4	0	0	4	5.24

Correlation between phenotypic and genotypic resistance

As shown in Table 10, the conformity rate between the resistance phenotype to tetracycline and the genotypic resistance was 82.83%, for macrolides it was 89.14% and for beta-lactams it was 95.80%.

Table-10. Results of the correlation analysis between antimicrobial resistance phenotypes and resistance genes from yak-derived streptococci in different regions of Tibet

<table>
<tr><th rowspan="2">Antibiotics group</th><th rowspan="2">Drug resistance genes</th><th colspan="2">Gene types</th><th rowspan="2">Phenotype of drug resistance</th><th rowspan="2">Coincidence rate</th></tr>
<tr><th>No. strains</th><th>Positivity rate</th></tr>
<tr><td rowspan="4">Tetracyclines</td><td>tetK</td><td>64</td><td rowspan="4">15.97%</td><td rowspan="4">19.28%</td><td rowspan="4">82.83%</td></tr>
<tr><td>tetL</td><td>69</td></tr>
<tr><td>tetO</td><td>55</td></tr>
<tr><td>tetM</td><td>92</td></tr>
<tr><td rowspan="3">Macrolides</td><td>ermA</td><td>183</td><td rowspan="3">44.16%</td><td rowspan="3">49.54%</td><td rowspan="3">89.14%</td></tr>
<tr><td>ermB</td><td>311</td></tr>
<tr><td>mefA</td><td>138</td></tr>
<tr><td>β-Lactams</td><td>pbp2b</td><td>25</td><td>5.24%</td><td>5.47%</td><td>95.80%</td></tr>
</table>

Discussion

The yak (Bos tibeticus), a distinct bovid species native to the Qinghai-Tibet Plateau, is a herbivorous ruminant that is typically found in cold regions of high altitudes above 2,500 meters (Waites et al., 2011; Yu R et al., 2022). As a significant source of income for Tibetan herders, yaks are often grazed freely, increasing the risk of transmission of diseases and the occurrence of various diseases (Gao et al., 2020). With numerous species of *Streptococcus* that pose significant threats, including *Streptococcus pneumoniae*, *Streptococcus bovis*, *Streptococcus suis*, and *Streptococcus agalactiae*, they can cause damage to yaks. The issue of antibiotic resistance is increasing, with multidrug resistance becoming more prevalent (Basit et al., 2023; Rasool et al., 2024). Therefore, it is crucial to understand the antibiotic resistance patterns of *Streptococcus* to implement appropriate treatment strategies, minimize overuse, and reduce drug residues, ultimately ensuring human health and food safety (Zhang et al., 2018).

In this study, 827 fecal samples of diarrhea cases were collected from yaks in Tibetan regions, and 477 *Streptococcus* isolates were isolated, with a total isolation rate of 57.68%. The highest isolation rate was in Changdu (71.43%), while the lowest was in Naqu (34.09%). A 12-antibiotic sensitivity test was conducted on 140 isolates of yak-origin *Streptococcus* from seven Tibetan cities. The results showed that the yaks in these regions had a high resistance rate to macrolides (Erythromycin and Midecamycin) at 52.86% and 52.14%, respectively. Resistance to tetracyclines (Tetracycline, Doxycycline, and Minocycline) was 25%, 18.57% and 14.29%, respectively. Aminoglycosides (Kanamycin, Gentamicin, Neomycin, and Streptomycin) had the lowest resistance rates of 8.57%, 6.43%, 5.00% and 8.57%, respectively. The β-lactam antibiotics (Cefotaxime, Cephazolin, and Carbenicillin) had the lowest resistance rates at 7.14%, 2.15% and 7.14%, respectively. The analysis revealed a significant presence of multidrug resistance, with 37 different resistance patterns identified, with Erythromycin/Midecamycin (ERY/MID) being the dominant. The study's findings indicate widespread resistance in *Streptococcus* of yak origin in Tibet, with an increasing trend of a single bacteria being resistant to multiple drugs. This highlights the need to diversify clinical antibiotic use, avoid macrolides such as Erythromycin and Midecamycin, and consider combined or alternating drug regimens.

The heat map, a visual representation of antibiotic sensitivity data, was generated using Origin 2021 to analyze 140 isolates of yak origin from seven Tibetan cities. The results showed a high resistance rate to macrolides and a relatively low resistance rate to β-lactam antibiotics. Antibiotic resistance genes are primarily responsible for bacterial resistance, with plasmids, integrons, and transposons being key transfer elements. In this study, eight resistance genes were selected based on the sensitivity test results, and a resistance gene detection was carried out on 477 isolates of yak origin from the seven regions. Eight genes were detected, with *ermb*, *erma*, and *mefa* (Macrolide resistance genes) having the highest detection rates at 65.20%, 38.36%, and 28.93%, respectively. *tetk*, *tetl*, *teto*, and *tetm* (Tetracycline resistance genes) had detection rates of 13.42%, 14.47%, 11.53%, and 20.34%, respectively, which were significantly higher than the findings reported by Zhang et al. (2018) (4.55%, 22.73%, 35.23%, and 75.00%). The lowest detection rate was for *pbp2b* (β-lactam resistance gene), at 5.24%. The compatibility rate between detected resistance genes and their corresponding antibiotics was found to be greater than 82.83%.

The study reveals severe antibiotic resistance in yaks in Tibetan regions, emphasizing the need for authorities to prioritize *Streptococcus* control and regulation. Veterinarians should consider the use of antibiotics with higher sensitivity rates, practice rational drug use, and employ combined or alternating treatments. Regular drug testing and monitoring should be implemented to prevent the emergence of superbugs, control the spread of *Streptococcus*, reduce antibiotic residues, and protect human health and food safety. More research is needed to explore the mechanisms of resistance to *Streptococcus* and identify alternative biological agents to combat the problem effectively.

Conclusion

In this study, 477 strains of *Streptococcus* were isolated and identified from 827 yak diarrheal fecal samples from seven cities in Tibet. The isolated stains showed resistance to a variety of drugs. The multi-drug resistance was serious, among which the ERY/MID mode was dominant, and the detection rate of drug resistance genes was also high. This study lays a data foundation for the air defense and treatment of streptococci in various regions of Tibet.

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Disclaimer: None.

Conflict of Interest: None.

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Contribution of Authors

Li Z: Executed the experiments of this study, data curation and writing the first draft of the article.
Huang J, Bai Z & Zheng Z: Helped to complete the relevant basic experiments and data collection.
Bianba Y & Pu C: Conducted various analyses and proofread the manuscript.
Suolang S: Supervised all the experiments and arranged the funding for equipment and chemicals for this study.

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