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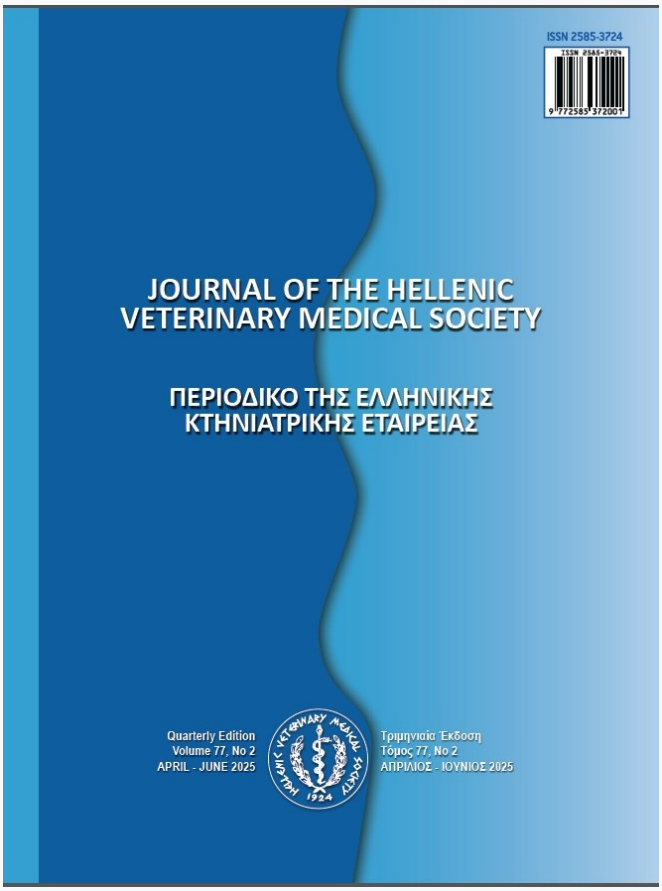

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Research article
Ερευνητικό άρθρο

Enterococcus faecium as a causative agent of death of Small-Tailed Han sheep farmed in Tongliao region of China: A case study

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ABSTRACT: *Enterococcus faecium* is a facultative anaerobic Gram-positive bacterium that exhibits high levels of tolerance (*Enterococcus faecium* can survive in harsh environments such as acidic, alkaline and high temperature environments). Some strains of *Enterococcus faecium* have been utilized as probiotics; however, certain strains of *Enterococcus faecium* are also considered pathogenic, with the potential to cause significant health concerns in humans and animals. *Enterococcus faecium* has demonstrated a notable capacity for acquiring resistant genes, thereby complicating its control and prevention. *Enterococcus faecium* disease is a significant infectious disease that affects the health of sheep. There is a paucity of reports on the subject of pathogenic *Enterococcus faecium* of sheep origin. In this study, we reported a strain of *Enterococcus faecium* isolated from a Small-Tailed Han sheep farm in Tongliao, Inner Mongolia Autonomous Region, China. We perform tests for isolation and culture of bacteria, *16S rDNA* identification, antimicrobial susceptibility test, detection of resistant genes, animal regression tests and observation of pathological sections. Tests confirm that the pathogen causing sheep deaths is *Enterococcus faecium* (GenBank sequence number: OP430848.1). Better growth of isolated strain on sheep blood agar medium was observed. The isolated strain exhibited 99.90% similarity to the *16S rDNA* fragment of *Enterococcus faecium* that has been published on GenBank. The antimicrobial susceptibility test revealed that florfenicol and amikacin exhibited the optimal bacteriostatic effect. Isolated strain containing *cfr*, *tetM* and *ant(6)-I* resistance genes. The results of animal regression tests demonstrated the high pathogenicity of the isolated strain. The histopathologic results indicated the presence of pathological changes in the substantial organs (heart, liver, spleen and kidney) of the deceased sheep. This study provides a reference point for the diagnosis, treatment, and investigation of drug resistance in pathogenic *Enterococcus faecium* of sheep origin. The future rational use of antibiotics in animal husbandry to curb the spread of resistant *Enterococcus faecium* is of great importance.

Keyword: Sheep; Enterococcus faecium; Isolation; Antibiotics; Resistance genes.

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INTRODUCTION

Enterococcus faecium is a Gram-positive bacterium that is a member of the genus *Enterococcus*. *Enterococcus faecium* is characterized by its ease of storage and resistance to acid and heat. It has been demonstrated that certain strains of *Enterococcus faecium* possess distinctive advantages in the domains of feed production and processing (Wang et al., 2020). These strains are frequently utilized as probiotics in the context of animal husbandry. Some *Enterococcus faecium* have been identified as pathogenic, with the capacity to be transmitted among humans and animals. Humans can become infected by ingesting food contaminated with fecal *Enterococci* of animal origin. The bacterium *Enterococcus faecium*, which is considered pathogenic, has been linked to various diseases in humans, including sepsis and pneumonia. A rising number of cases involving *Enterococcus faecium* have been documented in recent years (Yamaguchi et al., 2023; Chen et al., 2023; Wardal et al., 2022; Vogelaers et al., 2021). *Enterococcus faecium* infections have been documented in a variety of animals, including in kangaroos, pigs, sheep, chickens, musk deer, cattle, and foxes, among others (Chen et al., 2015; Grudlewska-Buda et al., 2023; Liu et al., 2017; Zhang et al., 2018; Gong et al., 2019; Wang et al., 2020; Wei et al., 2021). The infection is characterized by its high lethality rate in infected animals. Research has demonstrated that *Enterococcus faecium* possesses the capacity to disseminate resistance genes to other *Enterococci* via diverse mechanisms, thereby culminating in the augmentation of resistance in the latter (Liu, 2015; Zeng et al., 2023; Bai et al., 2021). *Enterococcus faecium* poses significant concerns for public health and the safety of feed production.

There is a paucity of reports regarding the occurrence of pathogenic *Enterococcus faecium* of sheep origin. As a pathogenic bacterium that can pose a threat to sheep health, the methods employed for the detection of *Enterococcus faecium* are subject to certain advantages and limitations. The traditional bacterial culture method is simple to operate, but the culture time is long and the growth of the test strain is easily interfered by other bacteria. The accuracy of the PCR assay is high, but the procedure of this method is cumbersome. Combined use of the results of multiple testing methods for evaluation can improve the accuracy of the diagnosis of *Enterococcus faecium*. The use of antimicrobial susceptibility testing allows for the screening of sensitive drugs for use in the treatment of *Enterococcus faecium*. In addition to antimicrobial susceptibility testing, detection of *Enterococcus faecium* resistance genes should be performed to optimize antibiotic use strategies. Rational selection of antibiotics for the treatment of *Enterococcus faecium* disease ensures efficacy and safety of treatment, thereby mitigating the impact of *Enterococcus faecium* on the sheep industry.

In this test, a strain of Gram-positive coccobacillus was isolated from the organs of dead Small-Tailed Han sheep and from the blood of Small-Tailed Han sheep (blood collected from non-dead diseased Small-Tailed Han sheep). The isolates were characterized by tests a variety of methods, including isolation and culture of bacteria, *16S rDNA* identification, antimicrobial susceptibility test, detection of resistance genes and observation of pathological sections. In addition, sensitive antibiotics were screened for the treatment of this bacterial disease. The aim of the study was to identify and characterize the agent responsible for the death of Small-Tailed Han sheep farmed in Tongliao region of China. This experiment provides a clinical reference and theoretical basis for the comprehensive prevention and control of this bacterial disease as well as for research on the pathogenesis of said disease.

MATERIALS AND METHODS

Sample origin and reagents

A Small-Tailed Han sheep farm has 1,800 sheep in Tongliao, Inner Mongolia Autonomous Region, China. In March 2022, 65 (26 ewes and 39 rams) of the 1,800 sheep were depressed and 27 sheep had died. Four types of tissues (heart, liver, spleen, and kidney) were collected from 27 deceased sheep at the Small-Tailed Han sheep farm in Tongliao for the purpose of serving as test materials. Additionally, ten samples of blood were also collected from the Small-Tailed Han sheep farm (blood collected from non-dead diseased sheep) as test materials. Subsequent to the demise of the sheep, organ collection was conducted in a sterile environment. The organs collected for histopathological examination were preserved in 10% formaldehyde solution, and the organs intended for microbiological examination were stored in sterile containers. Collection of blood samples was accomplished at the jugular vein of the diseased sheep using sterile blood collection tubes. After completing the collection of samples, the samples were transported to the laboratory for subsequent tests. LB agar medium (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium chloride 10 g L^{-1},

agar powder 15 g L^{-1}), LB broth medium (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium chloride 10 g L^{-1}), sheep blood agar medium (tryptone 23 g L^{-1}, starch 1 g L^{-1}, sodium chloride 5 g L^{-1}, agar powder 15 g L^{-1}, sterile defibrinated sheep blood 100 mL L^{-1}), a bacterial genomic DNA extraction kit, a viral genomic DNA/RNA extraction kit (centrifugal column type), a FastKing One Step RT-PCR Kit, a FastKing One Step RT-PCR cassette, nucleic acid dye GV-II, a DNA gel extraction kit, DL2000 DNA marker, and PCR master mix were purchased from Takara Biomedical Technology Co., Ltd., Beijing, China. The anaerobic agar medium and anaerobic broth medium were purchased from Qingdao Haibo Biotechnology Co., Ltd., Qingdao, China. Disk of antibiotics, agar powder, sodium chloride, yeast dipping powder, tryptone, Muller-Hinton broth medium, Muller-Hinton agar medium and sterile defibrinated sheep blood were purchased from Zhengzhou Dening Biotechnology Co., Ltd., Zhengzhou, China. *Brucella* selective medium was purchased from Oxoid Co., Ltd., Basingstoke, UK (this reagent was purchased by Shanghai Aiyan Biomedical Technology Co., Ltd., Shanghai, China). Colloidal gold test strips for peste des petits ruminant's virus, colloidal gold test strips for foot-and-mouth disease virus and colloidal gold test strips for *Brucella* were purchased from Shenzhen Finder Biotechnology Co., Ltd., Shenzhen, China. McFarland turbidimeter (Bacterial turbidimeter), McFarland turbidimeter tube were purchased from Shanghai Kunquan Biotechnology Company Shanghai, China. Twenty Kunming mice (6-8 weeks old, 25-30 g weight) were purchased from Liaoning Changsheng Biotechnology Co., Ltd., Benxi, China.

Methods

Detection of peste des petits ruminant's virus, foot-and-mouth disease virus, anaerobic bacteria and Brucella

A meticulous observation of the clinical signs exhibited by the infected sheep was conducted, and a series of tests for various infectious diseases with high lethality rates were performed to ascertain the underlying cause of death. The colloidal gold test strips were utilized to ascertain the presence of peste des petits ruminants' virus, foot-and-mouth disease virus, or *Brucella* in the sick sheep, in accordance with the prescribed protocol (a total of 27 organs from deceased sheep and 10 blood samples were utilized as test materials). Polymerase chain reaction (PCR) was employed to amplify the *N* gene of peste des petits ruminants' virus and the *VP1* gene of foot-and-mouth disease virus. This approach was adopted to determine whether the mortalities observed in the affected ovine population were attributable to infection with either virus. Anaerobic bacteria were cultivated using anaerobic agar medium (37 ℃, 24 h). Twenty-seven dead sheep organs and 10 sent blood samples were coated on *Brucella* selective medium and placed in a standard incubator without CO_2 and an incubator containing 7.5% CO_2 for 7 days at 37 ℃. The primer sequences are shown in Table 1 (Ge et al., 2008; Zhao, 2016). The PCR reaction system of peste des petits ruminant's virus (25 μL) was used with 12.5 μL 2×FastKing One Step RT-PCR master mix, 1 μL 25×RT-PCR enzyme mix, 0.75 μL of forward and 0.75 μL of reverse primers, 1 μL DNA template, and 9 μL ddH_2O. The reaction procedure was as follows: reverse transcription at 42 ℃ for 30 min, initial denaturation at 95 ℃ for 3 min; 36 cycles consisting of denaturation at 94 ℃ for 30 s, primer annealing at 61 ℃ for 30 s, and extension at 72 ℃ for 90 s; and a final extension at 72 ℃ for 10 min. The PCR reaction system (20 μL) for the *VP1* gene of foot-and-mouth disease virus was as follows: 10 μL 2×FastKing One Step RT-PCR master mix, 0.5 μL each of forward and 0.5 μL of reverse primers, 1 μL DNA template, 8 μL ddH_2O. The reaction procedure was as follows: reverse transcription at 42 ℃ for 30 min, initial denaturation at 95 ℃ for 3 min; 36 cycles consisting of denaturation at 94 ℃ for 30 s, primer annealing at 61 ℃ for 40 s, and extension at 72 ℃ for 90 s; and a final extension at 72 ℃ for 10 min.

Bacterial isolation culture

Observe the lesions in the organs sent for examination and then perform subsequent tests. Bacteria were collected from the organs of 27 dead sheep and 10 blood samples sent for examination using an inoculation loop in a sterile environment, and were inoculated into LB agar medium and sheep blood agar medium for incubation (37 ℃, 24 h). Following an observation of the characteristics exhibited by colonies, a single dominant colony was selected for Gram staining and microscopic observation. The anaerobic strains and isolated strains were cultured using anaerobic broth medium and LB broth medium for enrichment (37 ℃, 24 h), respectively.

DNA extraction and 16S rDNA sequence analysis of bacteria

A total of 1.5 mL of bacterial culture was taken, and bacterial DNA was extracted according to the steps of the DNA extraction kit (Takara Biomedical Tech-

Table 1. Primers used in the experiment.

Name of gene	Primer sequence (5′-3′)	Annealing temperature (°C)	Product length (bp)	Reference
PPRV-N	F: ATGGCGACTCTTCTTAAAAG R: CTAGCCGAGGAGATCCTTGT	60	351	Ge et al., 2008
VP1	F: GAAGGGCCCAGGGTTGGACTC R: GAGTCCAACCCTGGGCCCTTC	61	916	Zhao, 2016
16S rDNA	F: AGAGTTTGATCCTGGCTCA R: GGTTACCTTGTTACGACTT	55	1466	Zhao, 2016
ermB	F: CCGTTTACGAAATTGGAACAGGTAAAGGGC R: GAATCGAGACTTGAGTGTGC	58	359	Wang et al., 2018
cfr	F: TGAAGTATAAAGCAGGTTGGGAGTCA R: ACCATATAATTGACCACAAGCAGC	56	746	Zhang et al., 2018
tetM	F: ACAGAAAGCTTATTATATAAC R: TGGCGTGTCTATGATGTTCAC	52	171	Aminov et al., 2001
tetS	F: GAAAGCTTACTATACAGTAGC R: AGGAGTATCTACAATATTTAC	50	169	Aminov et al., 2001
ant(2″)-I	F: GAGCGAAATCTGCCGCTCTGG R: CTGTTACAACGGACTGGCCGC	50	320	Garofalo et al., 2007
ant(6)-I	F: ACTGGCTTAATCAATTTGGG R: GCCTTTCCGCCACCTCACCG	50	597	Li and Zhang, 2005
aac(6')/aph(2″)	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	50	220	Song et al., 2021
mefA	F: ACTATCATTAATCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	50	346	Zhang et al., 2012
parC	F: ATTGAATAAAGATGGCAATA R: CGCCATCCATACTTCCGTTG	54	191	Leavis et al., 2006

nology Co., Ltd., Beijing, China). Anaerobic strains and isolated strains were identified by PCR using universal primers for *16S rDNA* of bacteria, and the primer sequences are shown in Table 1 (Zhao, 2016). The PCR reaction system (20 μL) was utilized, comprising 10 μL of 2×DetPCR master mix, 0.5 μL of forward and 0.5 μL of reverse primers, 1 μL of DNA template, and 8 μL of ddH_2O. The reaction procedure was as follows: initial denaturation at 95 °C for 5 min; 35 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 52 °C for 1 min, and extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min.

The agarose gel products were then recovered and purified using a DNA gel recovery kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). Subsequently, the gel recovery products were dispatched to Shanghai Sangong Biotechnology Co., Ltd., (Shanghai, China) for the purpose of sequencing (the sequencing process was finalized using the Illumina Miseq, a second-generation sequencing platform). A comparison was made between the sequencing results and the sequences stored in the NCBI database (National Library of Medicine, Bethesda, USA). Constructing genetic evolutionary trees of genes using DNAstar software (version 7.1; DNAStar Inc., Madison, USA).

Antimicrobial susceptibility test

Bacteria were cultivated in Muller-Hinton broth medium for a period of 12 h. Take 100 μL of the bacterial culture and spread it on the Muller-Hinton agar medium. The susceptibility of the isolate to 10 commonly used antibiotics was determined using the K-B paper slide method according to the standards for antimicrobial susceptibility testing promulgated by the Clinical and Laboratory Standards Institute (Zimmer, 2024). The antibiotics used in this trial included oxazolidinones (Linezolid, 30 μg), aminoglycosides (amikacin, 30 μg; neomycin, 30 μg; ka-

namycin, 30 μg and gentamicin, 10 μg), macrolides (erythromycin, 15 μg), tetracyclines (florfenicol, 30 μg and tetracycline, 30 μg) and quinolones (enrofloxacin, 5 μg and ofloxacin, 5 μg).

Detection of resistance genes

Primers for the oxazolidinone resistance gene (*cfr*), tetracycline resistance genes (*tetM* and *tetS*), aminoglycoside resistance genes [*ant(6)-I*, *aac(6')/aph(2")* and *ant(2")-I*], erythromycin resistance genes (*ermB* and *mefA*), and quinolone resistance genes (*parC*) were synthesized with reference to the literature (Wang et al., 2018; Zhang et al., 2018; Aminov et al., 2001; Garofalo et al., 2007; Li and Zhang, 2005; Song et al., 2021; Zhang et al., 2012; Leavis et al., 2006). The primer sequences are delineated in Table 1. The PCR reaction system (25 μL) was as follows: 2×DetPCR master mix 12.5 μL, 1 μL of each forward and reverse primer, 1 μL of DNA template, and 9.5 μL of ddH_2O. The reaction procedure was as follows: initial denaturation at 95 ℃ for 5 min; 35 cycles consisting of denaturation at 94 ℃ for 1 min, annealing at a suitable temperature for 40 s, and extension at 72 ℃ for 90 s; and a final extension at 72 ℃ for 10 min (the *ermB* gene reaction procedure was as follows: initial denaturation at 94 ℃ for 5 min; 30 cycles consisting of denaturation at 94 ℃ for 45 s, primer annealing at 47 ℃ for 45 s, and extension at 72 ℃ for 1 min; and a final extension at 72 ℃ for 10 min).

Animal regression test and histopathological observations

Twenty mice were divided into two groups (ten mice per group). In the experimental group, each mouse was injected intraperitoneally with 0.1 mL of bacterial culture (adjustment of the concentration of the bacterial culture using the McFarland method, the concentration of the bacterial culture was 10^9 CFU mL^{-1}). The control mice were injected intraperitoneally with 0.1 mL of saline each (0.9%).

The organ tissue blocks (heart, liver, spleen and kidneys of infected and dead sheep) were immersed in 10% formaldehyde solution for 3 d for fixation. Pathological sections were prepared and then examined microscopically to observe the pathological changes in the tissues (tissue sections were stained using hematoxylin-eosin staining). In accordance with the protocols approved by the Institutional Animal Care and Ethical Committee of Inner Mongolia Minzu University, under the number 202007865, which adhere to the "Guidelines for ethical review of Laboratory Animal Welfare" (GB/T 35892-2018).

RESULTS

Results of testing for peste des petits ruminant's virus, foot-and-mouth disease virus, anaerobic bacteria and Brucella

The clinical symptoms of diseased sheep are shown in Table 2 (26 ewes and 39 rams). The results of

Table 2. Summary of clinical symptoms in Small-Tailed Han sheep

Symptoms classification	Symptomatic performance	Number of symptomatic sheep
Systemic symptom	Sick sheep are depressed and slow to respond. The diseased sheep's appetite is reduced or even abolished. Some of the sheep exhibited signs of fever.	All 65 diseased sheep exhibited symptoms of depression, slow response, as well as loss of appetite or even complete anorexia. Twenty-eight sheep exhibited signs of fever.
Digestive system symptoms	Diseased sheep show diarrhea symptoms, feces are yellowish green and thin paste, and the abdomen is sensitive to palpation.	Twenty - three diseased sheep exhibited digestive symptoms.
Respiratory system symptoms	Diseased sheep have difficulty breathing, cough, and cough up fluid containing blood.	Nineteen sick sheep showed respiratory symptoms.
Genitourinary system symptoms	Increased purulent vaginal discharge in ewes; red, swollen scrotum and sensitivity to palpation in rams.	Five out of the 26 diseased ewes manifested urogenital symptoms. Thirteen out of the 39 diseased rams exhibited urogenital symptoms.
Nervous system symptoms	The gait of the Small-Tailed Han sheep is unsteady, and some of them show signs of convulsions and coma.	Fourteen sick sheep showed neurological signs.

the colloidal gold test strips are plotted in Fig.1A. Colloidal gold test strips yielded a negative result (Fig.1B). PCR amplification of the *N* gene of peste des petits ruminant's virus and the *VP1* gene of foot-and-mouth disease virus did not amplify the target bands (Fig.1C). No Gram-negative bacilli suspected to be *Brucella* were not observed during bacterial culture. The combination of colloidal gold test results and bacterial culture results indicated that the sick sheep were not infected with *Brucella* (After 7 d of incubation at 37 °C, no yellow colony morphology was observed. *Brucella* can grow yellow colonies. We did not find yellow colonies after 7 d of incubation and therefore ruled out *Brucella* infection). A total of 37 strains of Gram-positive cocci were isolated from the organs of 27 deceased sheep and 10 blood samples that had been sent for examination in an anaerobic agar medium (Fig.1D and Fig.1E). The presence of peste des petits ruminants' virus, foot-and-mouth disease virus and *Brucella* was not detected in the organs of 27 deceased sheep nor in 10 blood samples sent for testing. Some of the pictures presenting the results are placed in Fig.1. A total of 37 Gram-positive cocci strains were isolated from 27 dead Small-Tailed Han sheep organs and 10 blood samples sent for examination using anaerobic agar medium. Due to the similarity in colony morphology and microscopic morphology of the 37 strains of Gram-positive cocci, pictures of some of the results are placed in Fig.1.

Results of bacterial isolation and purification

A thorough observation of the organs of the deceased sheep revealed slight hemorrhages in the heart and

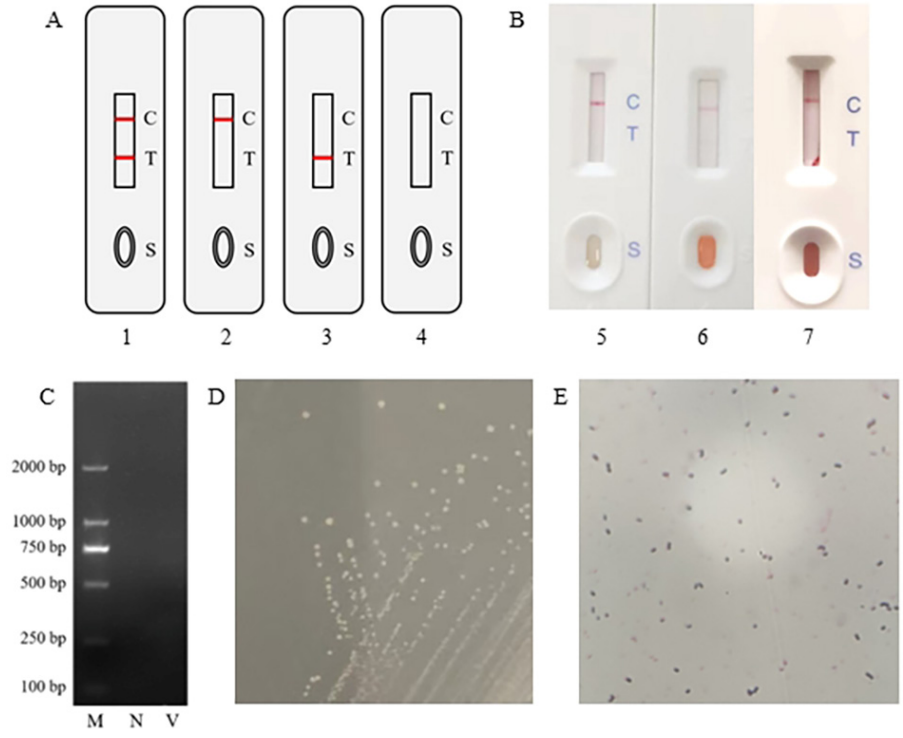

Figure 1. Results of testing for peste des petits ruminant's virus, foot-and-mouth disease virus, anaerobic bacteria and *Brucella*

Fig.1A Graph showing the effect of colloidal gold test strips. 1, positive test result; 2, negative test result; 3 and 4, invalid test. The "C" in the test card indicates the control line; the "T" indicates the test line; and the "S" indicates the sample addition hole.

Fig.1B Colloidal gold test strip results. 5, *Brucella* colloidal gold test strip results; 6, peste des petits ruminant's virus colloidal gold test strip results; 7, foot-and-mouth disease virus colloidal gold test strip results.

Fig.1C Results of amplification of *N* gene of peste des petits ruminant's virus and *VP1* gene of foot-and-mouth disease virus. M, DL2000 DNA marker; N, *N* gene; V, *VP1* gene

Fig.1D Anaerobic bacteria culture results.

Fig.1E Morphology of anaerobic bacteria under microscope (1 000×).

liver, and the kidneys exhibited signs of brittleness and visible foci (approximately the size of millet grains) on their surfaces. In this study, a total of 37 strains of Gram-positive cocci were isolated from 10 blood samples sent for examination and from the organs of 27 deceased sheep. The growth of bacteria incubated for 12 h was unsatisfactory. We extended the incubation time of the bacteria and observed them every hour. The results demonstrated that the bacterial samples were cultivated on LB agar medium for 24 h, resulting in the observation of visible colonies. The isolated strains grew white colonies on nutrient agar medium (Fig.2). On sheep blood agar medium, the bacteria grew into neatly-edged, creamy-white, smooth colonies, and there was no hemolytic ring around the colonies (Fig.2). Microscopic examination revealed that the isolate was Gram-positive cocci, predominantly arranged singly or in pairs (Fig.2). Due to the similarity in colony morphology and microscopic morphology of the 37 strains of Gram-positive cocci, some of the results are placed in Fig.2.

Sequence analysis results

Sequencing of PCR products showed that 37 anaerobic strains and 37 isolates were the same bacteria. The strain sequences were uploaded to the GenBank database (GenBank sequence number: OP430848.1). The results demonstrated a high degree of similarity between the isolated strain (OP430848.1) and the *16S rDNA* fragment of *Enterococcus faecium* issued on GenBank, with a percentage similarity of 99.90%. The 27th base of the measured *16S rDNA* sequence was mutated from T to A. Subsequent to this, the resulting sequences were aligned with analogous sequences in the GenBank database. The isolate was found to be in the same branch as the Beijing strain

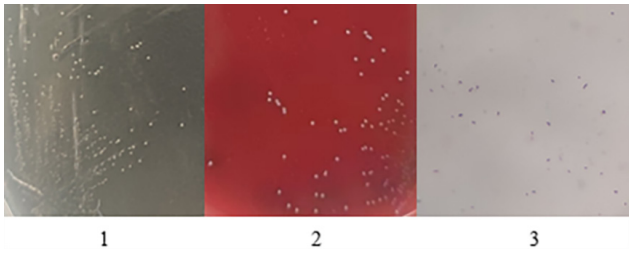

Figure 2. Culture and microscopic examination results of the isolated strains.
1, normal agar medium (37 °C, 24 h); 2, sheep blood agar medium (37 °C, 24 h); 3, morphology of bacterial isolate under microscope (1 000×).

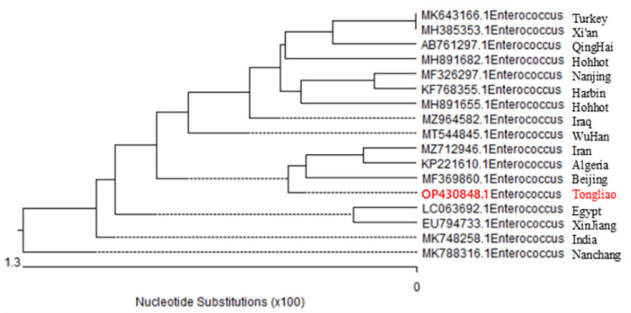

Figure 3. *16S rDNA* gene evolutionary tree.

(MF369860.1), the Iranian strain (MZ712946.1) and the Algerian strain (KP221610.1). The isolate demonstrated the closest genetic relatedness to the Beijing strain and was relatively genetically distant from the other virulent strains (Fig.3).

Antimicrobial susceptibility test results

A total of ten common clinical antimicrobial drugs were selected for the antimicrobial susceptibility test of the bacterial isolate (Table 3). The following antibiotics were found to be effective in inhibiting the growth of the isolates: amikacin, florfenicol, neomycin, tetracycline, enrofloxacin, ofloxacin, and gentamicin. The bacterial isolate exhibited resistance to linezolid, erythromycin, and kanamycin. Among the tested antibiotics, florfenicol and amikacin exhibited the most pronounced bacteriostatic effects. Consequently, the utilization of florfenicol or amikacin may be considered for the treatment of other diseased sheep on the Small-Tailed Han sheep farm.

Table 3. Results of antimicrobial susceptibility tests

Name of drug	Drug content of the pieces of paper/μg	Inhibitory ring diameter (mm)	Observed result
Linezolid	30	-	R
Amikacin	30	25	S
Erythromycin	15	-	R
Florfenicol	30	25	S
Neomycin	30	18	S
Kanamycin	30	8	R
Tetracycline	30	19	S
Enrofloxacin	5	18	S
Ofloxacin	5	18	S
Gentamicin	10	16	S

S, sensitive; I, Intermediate; R, resistant; -, no inhibitory ring.

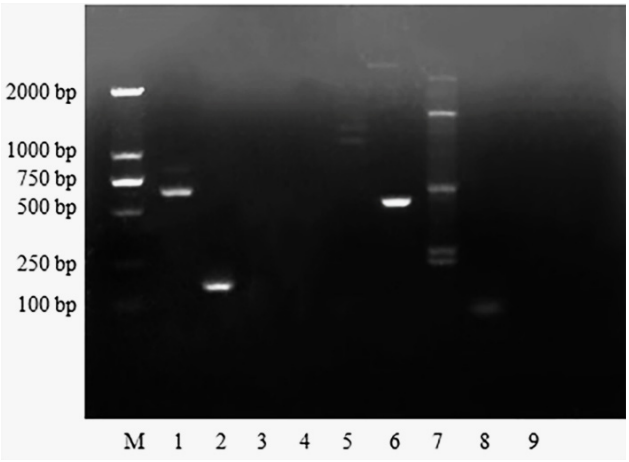

Figure 4. PCR amplification results of resistance genes of the isolated strain
M, DL2000 DNA marker;1, *cfr* gene; 2, *tetM* gene; 3, *tetS* gene; 4, *aac(6')/aph(2")* gene; 5, *ant(2")-I* gene; 6, *ant(6)-I* gene; 7, *mefA* gene; 8, *ermB* gene; 9, *parC* gene.

Resistance gene detection results

The isolated strains harbored the resistance genes *cfr*, *tetM*, and *ant(6)-I* (Fig.4).

Results of animal regression tests and pathological and histopathological observations

In the test group, 3 mice died within 6 h, and by 24 h, all the mice in this group had died. Histopathologic sections revealed significant hepatocyte necrosis, characterized by the loss of nuclei and the presence of vacuolation (Fig.5). A necrotic and nuclear loss phenomenon was observed in individual lymphocytes and macrophages in specific regions of the splenic red marrow (Fig.5). Glomerular endothelial cells were detached and necrotic, proximal tubules were severely necrotic, and the epithelium showed homogenized light staining (Fig.5). Individual myocardial fibers were swollen and fractured, with waxy necrosis and disappearance of cardiomyocyte nuclei (Fig.5).

DISCUSSION

Enterococcus faecium is a commensal bacterium that naturally colonizes the gastrointestinal tract of humans and animals. It is frequently utilized as a probiotic agent. In recent years, *Enterococcus faecium* has emerged as a conditionally pathogenic bacterium. Reports of *Enterococcus faecium* causing illness have been increasing on an annual basis (Graham et al., 2007). In this study, bacteria isolated from organs were cultured, and microscopic examination revealed that they were single or paired Gram-positive cocci. The *16S rDNA* identification indicated that the isolate was *Enterococcus faecium*. The colloidal gold test strips for foot-and-mouth disease virus, peste des petits ruminants' virus, and *Brucella* were all found to be negative. The PCR amplification targeting the *VP1* gene of foot-and-mouth disease virus and the *N* gene of peste des petits ruminants' virus did not yield any target bands. It was observed that the infected and deceased sheep were not infected with the aforementioned three pathogens. The initial investigation determined that the cause of death in the sheep was due to an *Enterococcus faecium* infection. The 27th base of the *16S rDNA* sequence measured in this study was mutated from T to A. The measured sequences were subsequently translated into protein sequences. The results demonstrated no alterations in the protein sequence. The association between base mutations and bacterial pathogenicity remains to be elucidated through further study and analysis.

The distribution of resistant bacteria in the environment is a consequence of the unregulated use of antibiotics (Zhang et al., 2012; Allel et al., 2023). *Enterococcus faecium* exhibits resistance to the ma-

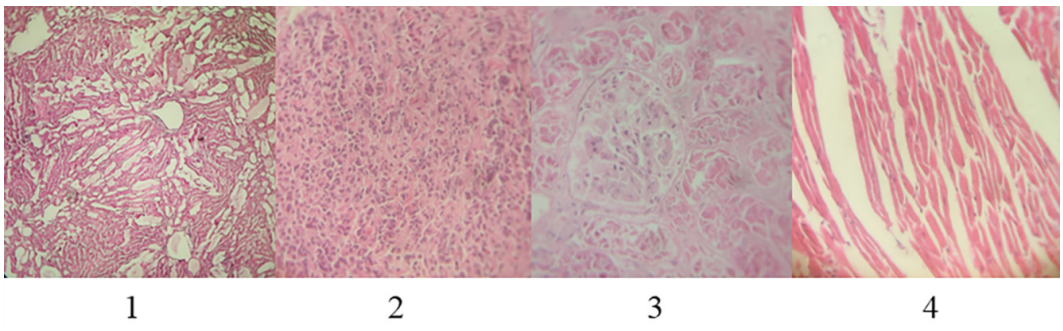

Figure 5. Pathologic sections of organs.
1, liver section (100×); 2, spleen section (100×); 3, kidney section (400×); 4, heart section (100×).

jority of commonly utilized antibiotics, thereby complicating the prevention and control of this bacterium (Xiao et al., 2019; Agga et al., 2022). Consequently, the judicious selection of appropriate antibiotics is imperative to manage this bacterial disease. Antimicrobial susceptibility testing showed that florfenicol and amikacin were relatively effective in inhibiting the isolated strain. One of these two antibiotics can be used to treat sheep infected with *Enterococcus faecium* on the Small-Tailed Han sheep farm. The results of the resistance gene assay showed that the isolate strain contained three resistance genes, *cfr*, *tetM*, and *ant(6)-I*, and did not contain *ermB*, *tetS*, *ant(2")-I*, *aac(6')/aph(2")*, *mefA*, or *parC* resistance genes. The bacteria exhibited a negative result for the partial resistance gene; however, they demonstrated resistance to the antibiotic. For instance, the erythromycin resistance genes (*mefA* and *ermB*) were found to be negative, yet the isolate demonstrated erythromycin resistance. Bacteria tested positive for some of the resistance genes, but the bacteria are still sensitive to antibiotics. For instance, the *ant(6)-I*, an aminoglycoside resistance gene, exhibited a positive result; however, the isolate demonstrated susceptibility to aminoglycosides (amikacin, neomycin, and gentamicin). The tetracycline resistance gene *tetM* exhibited positive results; however, the isolate demonstrated sensitivity to tetracycline. In summary, it is possible that other genes of the same drug class mediate bacterial resistance, or that the genes remain silent in a particular environment, thus leading to both of these phenomena (resistance genes can directly or indirectly affect the expression of proteins, which in turn affects the bacteriostatic effect of antibiotics). Research has identified a significant correlation between bacterial resistance and the formation of bacterial biofilms. The formation of biofilms has been demonstrated to enhance bacterial resistance and transmission (Zhang et al., 2018; Lu et al., 2014). The escalating virulence and resistance exhibited by *Enterococcus faecium* necessitate heightened preventive measures and control strategies to mitigate the repercussions of this bacterial disease on production.

Pathogenic *Enterococcus faecium* can pose a serious threat to human and animal health and can even be life-threatening. *Enterococcus faecium* can be transmitted in a variety of ways, either directly from animals to humans or indirectly through media such as food or water. *Enterococcus faecium* is highly capable of acquiring resistant genes, and it can spread resistant genes between different bacteria through genetic elements such as plasmids and transposons. This property poses a significant challenge to the clinical treatment of *Enterococcus faecium*. *Enterococcus faecium* have a significant impact on public health and safety and certain measures should be taken to prevent *Enterococcus faecium* infections. Timely disinfection of animal enclosures, breeding equipment, etc., and timely cleaning of feces and sewage, thereby reducing the reproduction and spread of *Enterococcus faecium*. If there are already animals showing signs of suspected infection, they need to be immediately isolated in groups and treated with antibiotics in a rational manner. It is imperative to provide professional training to farming personnel to enhance their knowledge of *Enterococcus faecium* and their capacity to prevent and control it. Moreover, standardizing the operational procedures in production is essential. The aforementioned measures have been demonstrated to be effective in reducing the risk of *Enterococcus faecium* infection. The utilization of *Enterococcus faecium* as a feed or food additive must be preceded by a thorough characterization of the biological properties of the strains employed, ensuring the safety and stability of the strains utilized. The majority of research on *Enterococcus faecium* has centered on the detection of resistance genes and the assessment of antibiotic susceptibility. The increasing resistance of *Enterococcus faecium* is a consequence of the indiscriminate use and abuse of antibiotics. To prevent the emergence of drug-resistant bacteria, researchers should develop new alternatives to antibiotics to slow the development of resistance. Biofilm formation enhances bacterial resistance, and inhibition of biofilm growth indirectly prevents the development of resistance in bacteria. Further exploration is necessary to elucidate the resistance mechanism of *Enterococcus faecium*.

CONCLUSION

In this experiment, a bacterial strain (GenBank accession number: OP430848.1) was isolated from diseased and dead sheep at a Small-Tailed Han sheep farm in Tongliao, China. The isolate was identified as *Enterococcus faecium* through conventional bacterial isolation and molecular biology testing methods. A screening of *Enterococcus faecium* for resistance genes and antimicrobial susceptibility tests determined that amikacin or florfenicol could be used clinically to treat other diseased sheep infected with *Enterococcus faecium* on the Small-Tailed Han sheep farm.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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