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NOTE

Bacteriology

Identification and pathogenicity analysis of *Streptococcus equinus* FMD1, a beta-hemolytic strain isolated from forest musk deer lung

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ABSTRACT. *Streptococcus* spp. cause a wide range of diseases in animals and humans. A *Streptococcus* strain (FMD1) was isolated from forest musk deer lung. To identify the bacterium at the species level and investigate its pathogenicity, whole genome sequencing and experimental infections of mice were performed. The genome had 97.63% average nucleotide identity with the *S. equinus* strain. Through virulence gene analysis, a beta-hemolysin/cytolysin genome island was found in the FMD1 genome, which contained 12 beta-hemolysin/cytolysin-related genes. Hemolytic reaction and histopathological analysis established the strain's pathogenicity in mice. This is the first report of a beta-hemolytic *S. equinus* strain in forest musk deer identified based on phenotypic and genotypic analyzes; this strategy could be useful for analyzing pathogens affecting rare animals.

KEY WORDS: beta-hemolytic, forest musk deer, *Streptococcus equinus*, whole genome analysis

Forest musk deer (*Moschus berezovskii*) is a medium-sized mammal that inhabits alpine forests. This animal has a high economic value because of the musk secreted by adult males, which plays an important role in traditional Asian medicine and the international perfume industry [23]. Because of the wild origin of the forest musk deer, it is difficult for people (including breeders) to approach the animal, thus, noticing the onset of diseases is also difficult. In December 2018, a 10-year-old forest musk deer at the Sichuan Institute of Musk Deer Breeding (Chengdu, China) suddenly fell down, showed anorexia and purulent nasal secretion, and died before the veterinarian's arrival. At autopsy, it was observed that the lungs were severely swollen, covered with petechial hemorrhages, and surrounded by a yellow peptone-like exudate (Supplementary Fig. 1). The lung tissue was collected and transported on ice to the laboratory for bacterial examinations.

First, the lung was streak-inoculated onto 5% sheep blood agar plates under aerobic and anaerobic conditions and incubated for 20 hr at 37°C. The single colony was selected and placed on 5% sheep blood agar plates based on the morphological characteristics. Then, the pure culture was subjected to Gram staining and biochemical identification. After this, the total DNA of the isolate was used as a template for a polymerase chain reaction (PCR) to amplify the 16S rRNA region with universal primers 27F and 1492R. The PCR procedure was the same as that described in a previous report [24]. The sequencing results were compared with the reference sequences from the NCBI database via BLAST. For the phylogenetic analysis, a dataset of 16S rRNA gene sequences was built, and all reference sequences were extracted from GenBank with high similarity based on the results of NCBI BLAST. The program used for the phylogenetic analysis was the same as that used in a previous article description [24]. In addition, the genome sequence of the isolate was sequenced at Novogene Bioinformatics Technology Co., Ltd., Beijing, 100000, China. The average nucleotide identity (ANI) and virulence genes of the isolate were identified using an ANI calculator (http://enve-omics.ce.gatech.edu/ani/index) [8] and virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) [12], respectively. The names of the virulence genes were determined using NCBI BLASTn (e-value <1e-10, identity >80%, and coverage >90%) [21]. The complete genomes of *S. equinus*, *S. lutetiensis*, and *S. infantarius* were used as reference genomes for the ANI analysis.

Twelve BALB/c mice were divided into two groups: test and control. All experiments using mice were approved by the committee

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for the animal experimentation of the Sichuan Agricultural University. The bacterial suspension was diluted to 4.3 × 10^8 CFU/m*l*, and six mice were intraperitoneally injected with 0.2 m*l* of the bacterial suspension. At the same time, the control group was injected with physiological saline. The infected and healthy mice groups were sacrificed after seven days, and then the lungs were immersed in 4% neutral buffered formalin and stained with hematoxylin and eosin for pathological observation. Meanwhile, the lung tissue of dead forest musk deer was processed as outlined above.

After 20 hr of incubation, smooth, grayish, and neat-edged colonies were found with a small zone of clear beta-hemolysis on the 5% sheep blood agar plates under both aerobic and anaerobic conditions (Fig. 1). Gram staining results showed that the isolate was a Gram-positive, round-shaped bacterium. Moreover, the isolate was negative in mannite, sorbitol, aesculin, inulin, catalase, and trehalose, positive in glucose, lactose, raffinose, and salicylic acid, and could not grow in Trypticase Soy Broth supplemented with 6.5% (w/v) NaCl. Electrophoresis indicated that the size of the PCR product of the 16S rRNA gene was 1,452 bp (Accession No. MK652875), which was highly similar (Ident >99.45%, Query cover=99%) to the 16S rRNA sequences of *S. equinus*, *S. lutetiensis,* and *S. infantarius*. In addition, the FMD1 strain was alone in a clade in the phylogenetic tree based on the 16S rRNA sequences of 60 Streptococci species (Fig. 2). Based on the above-mentioned characteristics, the FMD1 strain was difficult to identify at the species level based on conventional methods and 16S rRNA sequence analysis.

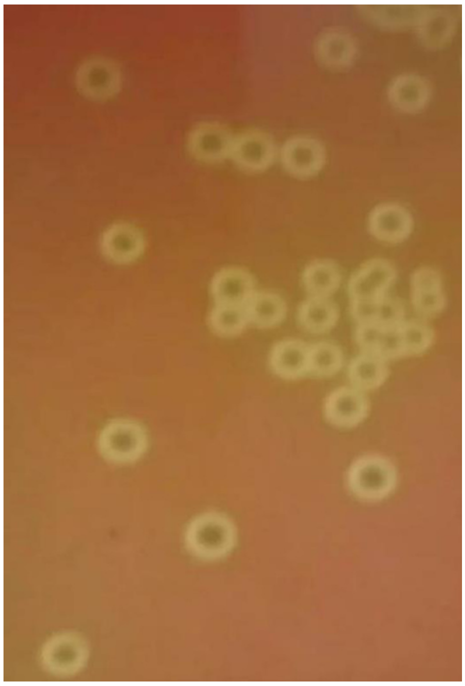

Fig. 1. Hemolytic phenotype analyses of the *Streptococcus equinus* FMD1 strain on 5% sheep blood agar plates.

Streptococcus is a diverse genus, encompassing approximately 77 species of bacteria, which infect a host of different animals [9]. Many Streptococci species are well-known pathogens in humans and animals, including *S. pneumoniae*, *S. suis*, and *S. equinus* [9, 11], and cause a broad range of diseases [5]. In contrast, *S. gallolyticus* and *S. infantarius* play important roles in traditional fermented food products across the world [18]. Hence, there is a potential public health risk if Streptococci species are not accurately identified.

To better understand the species level and pathogenicity of the isolated *Streptococcus* strain, the genome sequence was used for ANI and virulence gene analysis. The whole genome sequence of FMD1 was submitted to GenBank under the accession number SPDR00000000. The FMD1 genome was aligned with the *S. equinus* NCTC8140 strain assemblies at 97.63% ANI, while the highest ANI with *S. lutetiensis* and *S. infantarius* was 87.15% and 86.95%, respectively. The ANI analysis results identified the FMD1 strain as *S. equinus*.

Conventional methods and PCR analyzes have been developed to improve the species identification of bacteria [2]. However, some studies have encountered challenges in identifying the bacterial species isolated from patients, such as *Clostridium*, *Yersinia*, *Klebsiella* and *Streptococcus* [10, 13, 15, 16]. The accurate identification of bacteria at the species level has become increasingly more important [6]. Thus, many reviews highly recommended whole genome sequencing and analysis to increase the confidence in the species identification accuracy [4]. ANI is a useful tool, used to improve the accuracy of bacterial identification, and has been proposed as the best option for determining species [4, 22].

Notably, in the FMD1 genome Scaffold 2, a beta-hemolysin/cytolysin genome island (SPDR01000002: 58,344–69,620) was found, which contains 12 beta-hemolysin/cytolysin related genes (Table 1), including *cylX*, *cylD*, *cylG*, *acpC*, *cylZ*, *cylA*, *cylB*, *cylE*, *cylF*, *cylI*, *cylJ*, and *cylK*. The gene structure agreed with the beta-hemolytic genome island, which was found in group B Streptococci and contributed to disease pathogenesis [3]. To the best of our knowledge, a beta-hemolysin *S. equinus* strain has been isolated from bovine milk by Babu [1]. Previous studies [14, 17] have reported that hemolytic reaction is a strong evidence of the pathogenic potential of microorganisms, and beta-hemolysin may direct tissue injury or the activation of the host inflammatory response. As an important intestinal bacterium, *S. equinus* was first isolated in 1906, was frequently detected in feces, and thought to be a nonpathogenic bacterium for a long time [19]. In recent years, some diseases caused by *S. equinus* have been reported [7]. This study is the first report of a beta-hemolytic *S. equinus* strain in the forest musk deer.

Forest musk deer is a first-class protected animal in China, and therefore animal experimentation using this species is forbidden. We have successfully established a mouse challenge model of the *Escherichia coli* O78 strain and established a foundation for future research on the pathogenesis of pathogens isolated from forest musk deer [20]. To better understand the pathogenesis of *S. equinus* FMD1, mice are an excellent experimental animal for replacing forest musk deer. The pathological features of the lungs of mice and forest musk deer showed different degrees of histopathological changes (Fig. 3). The histological lesions in the lung tissues of mice and forest musk deer showed infiltration of numerous erythrocytes and inflammatory cells in the alveolar lumen. The alveolar epithelial cells of mice proliferated, but there was no standard for the alveolar walls of healthy forest musk deer. Lung histological lesions and hemolytic reactions confirmed that the *S. equinus* FMD1 strain is a pathogenic bacterium affecting mice and forest musk deer.

Our results indicate that whole genome analysis is a useful strategy for improving the accuracy of bacterial identification and

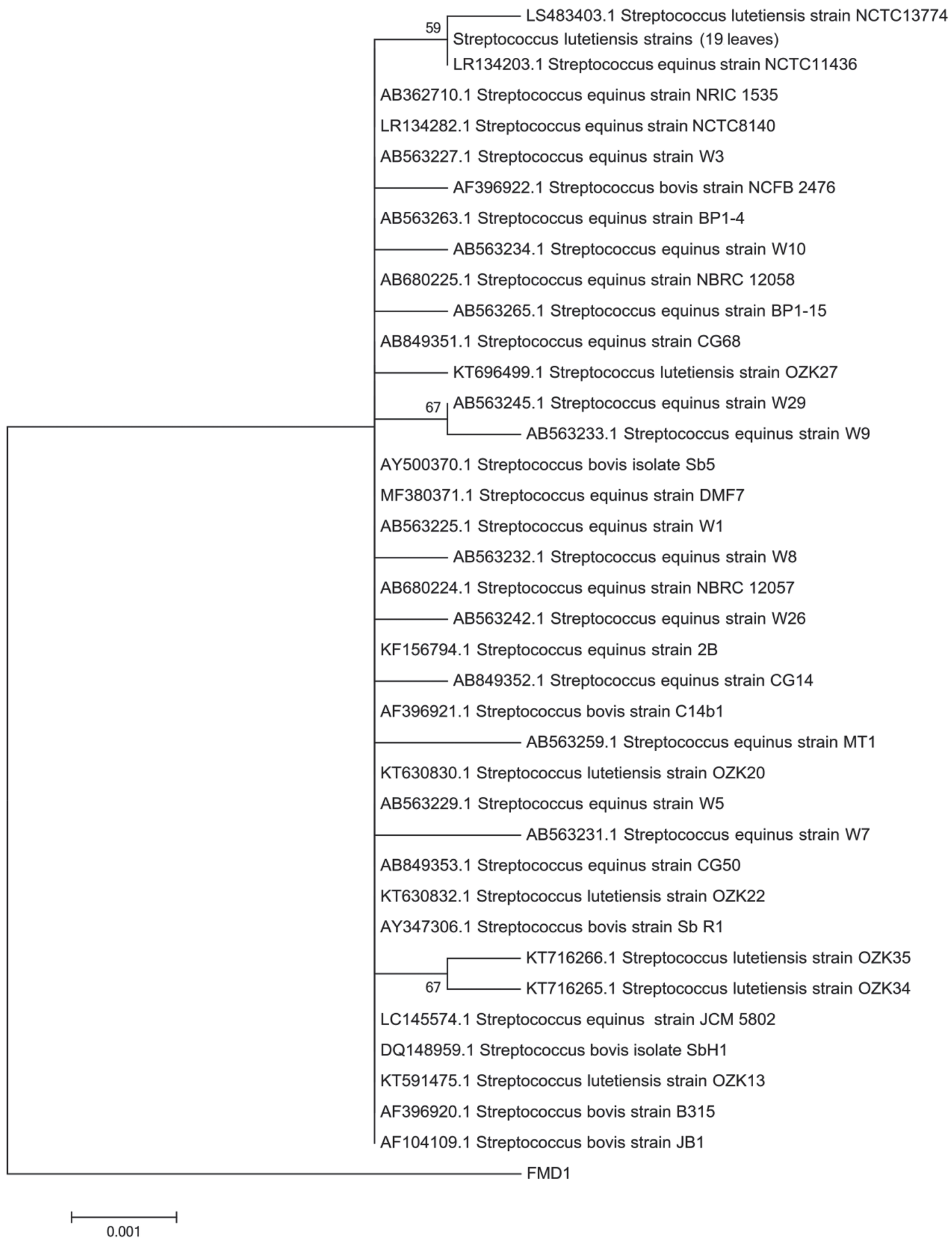

Fig. 2. Phylogenetic comparisons of 60 Streptococci strains using 16S rRNA gene sequencing. This phylogenetic tree includes 7 *Streptococcus bovis* 16S rRNA gene sequences, 23 *Streptococcus equinus* 16S rRNA gene sequences, 3 *Streptococcus infantarius* 16S rRNA gene sequences, and 26 *Streptococcus lutetiensis* 16S rRNA gene sequences, along with the FMD1 16S rRNA gene sequence. Multiple sequence alignments were performed using Clustal X 2.1. The phylogenetic tree was constructed using the neighbor-joining method with the MEGA 6.0 program. Genetic distances were determined using Kimura's 2-parameter model. The robustness of individual branches was estimated using bootstrapping with 1,000 replications. The scale bar corresponds to 0.001 estimated nucleotide substitutions per site.

mining genetic information. To the best of our knowledge, this report is the first to describe the beta-hemolytic *S. equinus* strain in forest musk deer. However, additional laboratory research investigating the beta-hemolytic genome island of the FMD1 strain is warranted.

Table 1. Genetic constitution of the beta-hemolysin/cytolysin genome island in the *Streptococcus. equinus* FMD1 strain

Orf ID	Name	Identity (%)	CDS region in nucleotide	VFDB_ID	Product
Orf01544	*cylX*	96.44	SPDR01000002:58344–58652	VFG005761	Acetyl coenzyme A CoA carboxylase CylX
Orf01545	*cylD*	98.22	SPDR01000002:58649–59494	VFG005764	Malonyl-CoA-ACP transacylase CylD
Orf01546	*cylG*	97.65	SPDR01000002:59491–60213	VFG005766	CylG protein
Orf01547	*acpC*	99.34	SPDR01000002:60206–60508	VFG005770	AcpC acyl carrier protein AcpC
Orf01548	*cylZ*	97.69	SPDR01000002:60495–60971	VFG005773	3R-hydroxymyristoyl ACP dehydratase CylZ
Orf01549	*cylA*	98.28	SPDR01000002:60961–61890	VFG005776	ABC ATP-binding cassette transporter CylA
Orf01550	*cylB*	98.63	SPDR01000002:61883–62761	VFG005779	ABC ATP-binding cassette transporter CylB
Orf01551	*cylE*	97.96	SPDR01000002:62758–64725	VFG005780	CylE protein
Orf01552	*cylF*	98.32	SPDR01000002:64728–65678	VFG005785	Aminomethyltransferase CylF
Orf01553	*cylI*	97.67	SPDR01000002:65678–67870	VFG005788	Putative 3-ketoacyl-ACP synthase CylI
Orf01554	*cylJ*	97.08	SPDR01000002:67880–69106	VFG005790	CylJ protein
Orf01555	*cylK*	97.44	SPDR01000002: 69114–69620	VFG005793	CylK protein

Orf, open reading frame; CDS: coding sequence; VFDB: virulence factors database.

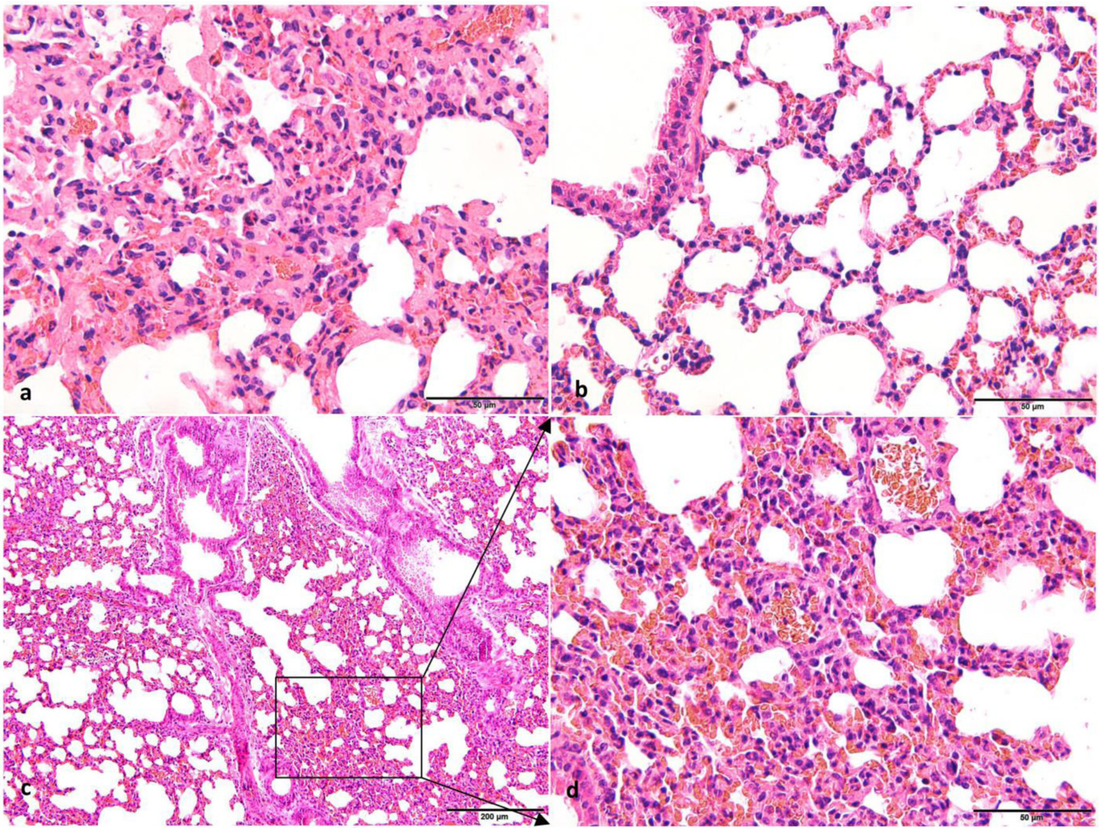

Fig. 3. Photomicrographs of lung tissues from mice and forest musk deer. (a) The histological structure of the lung of forest musk deer (H&E. 400×, Bar=50 μm). (b) The histological structure of the lung in the control group mice (H&E. 400×, Bar=50 μm). (c) The histological structure of the lung in the test group mice (H&E. 200×, Bar=200 μm). (d) The histological structure of the lung in the test group mice with high-expansion (H&E. 400×, Bar=50 μm). Histopathological observations showed infiltration of numerous erythrocytes, neutrophils, and monocyte in the alveolar lumen of forest musk deer and test group mice. In addition, an area of thickened alveolar wall was observed in the test group mice. There were no histopathological changes in the control group.

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