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SHORT COMMUNICATION

Identification of a Novel Genotype of *Streptococcus equi* Subspecies *equi* in a Donkey Suffering from Strangles

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ABSTRACT

Strangles is a contagious bacterial disease of equines and is prevalent worldwide. Host-restricted *Streptococcus equi* subspecies *equi* is the etiology. In April 2019, strangles was diagnosed in a donkey in the quarantine area of an intensive donkey farm. The infection was confirmed by clinical signs, bacterial isolation, histochemistry, PCR and sequencing. Based on the M-protein typing method, analysis of SeM sequencing data indicated that the isolated *S. equi* strain CN190402 belongs to a novel genotype SeM-138. The present study increases knowledge of the molecular epidemiology of *S. equi* and will lead to a greater understanding of the diversity of this pathogen. Meanwhile, it emphasized the necessity of quarantine areas and the importance of biosecurity for intensive donkey farms to prevent future outbreaks.

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INTRODUCTION

Strangles is a contagious bacterial disease of equines and is prevalent worldwide and caused by host-restricted *Streptococcus equi* subspecies *equi* (Harrington *et al.*, 2002). Clinical signs of strangles include fever, pharyngitis, dyspnea, profuse mucopurulent nasal discharges, anorexia, abscessation in the submandibular and retropharyngeal lymph nodes, and occasionally more severe internal illness and death in equids (Boyle *et al.*, 2018). Since strangles was first reported in 1251 (Slater, 2003), it has caused huge economic losses in the equine industry and formed a considerable threat to equine welfare worldwide.

S. equi can spread easily through normal equine social behavior including nose-to-nose contact and contaminated facilities, equipment, feed and water sources (Boyle *et al.*, 2018). Strangles can be introduced when new equids carrying the infection are brought into the farm. During recent years, donkey farming has been increased tremendously in China due to the increased consumption of milk, meat, and a gelatin product prepared using donkey skin, i.e. Ejiao. Since the reproductive cycle of donkeys is longer than other domestic animals, the intensive donkey farms usually import donkeys from other regions thereby enhancing the chances of transmission of infectious diseases, including strangles.

In early April 2019, strangles was diagnosed in a donkey in the quarantine area of an intensive donkey farm. The infection was confirmed by clinical signs, bacterial isolation, Gram staining, histochemistry (methylene blue stain), PCR and sequencing. Based on the M-protein typing method (Kelly *et al.*, 2006), SeM sequencing data analysis indicated the involvement of isolated *S. equi* strain CN190402 belongs to a novel genotype.

MATERIALS AND METHODS

The affected donkey was imported from approximately 800 km away and then put into a quarantine area. Clinical signs of mucopurulent nasal discharge were found on the third day following importation. Nasal discharge via swab was collected on the fifth day when the donkey showed lacrimation and a

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profuse mucopurulent nasal discharge (Fig. 1a). The sample was immediately transported to the laboratory.

Isolation of bacteria and β-hemolytic tests were carried out using 5% blood agar (Hopebio, Qingdao, China). Isolated colonies with beta-hemolysis were examined by Gram and methylene blue staining.

DNA was extracted from the nasal discharge sample and a β-hemolytic colony utilizing the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Using ASW73 and ASW74 as primers, PCR was performed for the detection of the SeM gene of *S. equi* (Kelly *et al.*, 2006). The PCR products were purified consuming the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and TA-subcloning was perform with pMD19-T (Takara, Dalian, China). Subsequently, the purified PCR product and recombinant plasmid were subjected to Sanger sequencing using an ABI 3730XL sequencer at Sangon (Qingdao, China).

Data of sequencing were gathered using SeqMan software in the Lasergene Package (DNA-STAR Inc.) and analyzed using BLAST (NCBI). By using Clustal W, nucleotide sequence alignment was done. Sequences of all 137 SeM alleles were downloaded (PubMLST-SeM; https://pubmlst.org/szooepidemicus/seM/). Reference strain *S. equi* strain 4047 was used for the analysis of SeM alleles. By the neighbor-joining method using MEGA 7, a phylogenetic tree was assembled (Kumar *et al.*, 2016).

RESULTS AND DISCUSSION

Methylene blue stain was carried out for the pus sample from the mucopurulent nasal discharge. Chains of *S. equi* were observed microscopically (Fig. 1b). β-hemolytic colonies (Fig. 1c) obtained from the nasal discharge (pus) were Gram-positive (Fig. 1d).

The 502 bp 5' region of the SeM gene was successfully amplified by PCR. Nucleotide sequence alignment of PCR product and subcloning plasmid showed 100% identity. BLAST analysis indicated that the PCR product was a segment of SeM gene of *S. equi*. Therefore, the infection of this donkey with *S. equi* was confirmed.

Comparing with equivalent N-terminal variable regions from all SeM alleles available in databases (Kelly *et al.*, 2006), identified that the 5' SeM sequence of the CN190402 strain (GenBank accession number MK988511) contained a novel SeM allele, i.e., SeM-138 (https://pubmlst.org/szooepidemicus/seM/). Phylogenetic analysis showed that CN190402 was clustered into a group with SeM-88 and SeM-136, closely related to the SeM-136 that was associated with a strangles outbreak in an intensive donkey farm located in middle-east of China during 2019 (Dong *et al.*, 2019). Comparison of the nucleotide sequences among SeM-3 (4047 strain; GenBank FM204883), SeM-88, SeM-136, and SeM-138 identified that SeM-138 shares 94.2, 96.6 and 98.5% identities, respectively. Similarly, amino acid sequences comparison exhibited SeM-138 shares 90.8, 90.8 and 95.4% identities and 10, 10 and 5 amino acid variations, respectively (Table 1).

A variety of typing methodologies are being utilized for the epidemiological analysis of bacterial pathogens (van Belkum *et al.*, 2007). Multilocus sequence typing (MLST) was wildly used for typing bacteria, but this method does not adequately discriminate different strains of *S. equi* (Webb *et al.*, 2008). Single locus sequence typing (SLST) methods have been successfully used to study *Streptococcus pyogenes* by *emm* typing (Enright *et al.*, 2001) and *Staphylococcus aureus* using the protein A gene (Omer *et al.*, 2014). The SeM of *S. equi* is an anti-phagocytic sortase-processed surface protein that binds IgG and fibrinogen, is thought to be a key virulence factor (Meehan *et al.*, 2009). It was proved that SLST based on the hypervariable 5' portion of SeM can be exploited as an epidemiological tool to study the transmission of different *S. equi* strains (Kelly *et al.*, 2006). Based on the SeM typing method, 137 genotypes (alleles) have been identified on the SeM database. In the present study, a novel genotype of SeM-138 was identified, increasing our knowledge of the molecular epidemiology of *S. equi* and leading to a greater understanding of the diversity of this pathogen. In particular, the differences between CN190402 and the previously reported SeM-136 strain of *S. equi*, provide evidence that a distinct population of *S. equi* circulates within donkey populations in China.

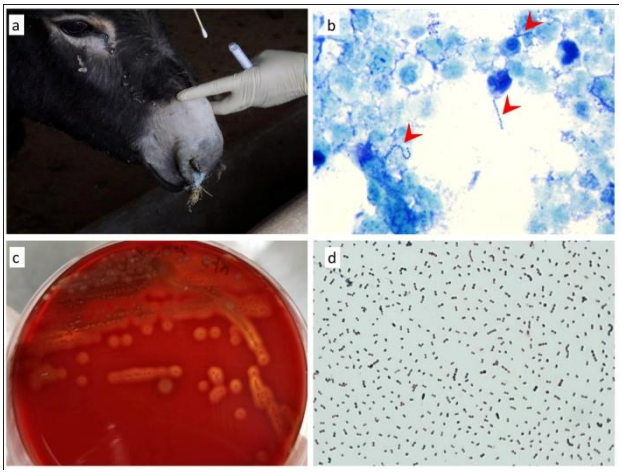

Fig. 1: Clinical signs, isolation and microscopic examination. (a) Mucopurulent nasal discharge; (b) Cocci arranged in chains (arrow-heads) - Methylene blue staining; (c) β-hemolytic colonies of *S. equi* on blood agar; (d) Isolated bacteria showed Gram-positive cocci (Gram staining). b and d = Oil lens - 100×.

Table 1: Amino acid sequences of reference strain 4047 (SeM-3) (FM204883), SeM-88 allele, SeM-136, and SeM-138 (CN190402)

Codon # of SeM	Allele (strain)			
	SeM-3 (4047)	SeM-88	SeM-136	SeM-138 (CN190402)
47	R	T	R	R
52	L	F	F	F
57	S	N	S	S
58	E	E	V	L
62	S	T	T	T
63	R	G	R	R
65[a]	-	H[a]	-	-
70	K	K	E	K
81	G	R	S	S
103	Y	Y	Y	H
107	V	I	I	R
108	H	Q	Q	Q
113	L	P	P	P
122	R	S	S	S
125	S	N	N	K
127	A	S	A	A

[a]Insertion in SeM-88 allele.

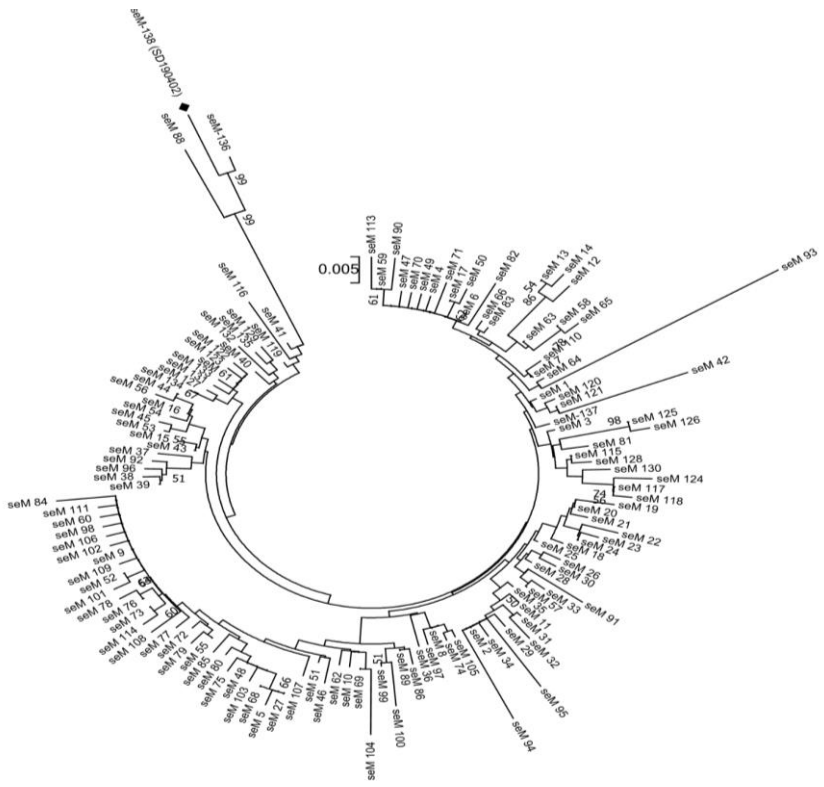

Fig. 2: Phylogenetic tree of SeM-138 (◆) and other alleles. The evolutionary history was inferred using the Neighbor-Joining method based on the variable N-terminal region of the seM gene. Evolutionary analyses were conducted in MEGA7. The number on the nodes shows the percent occurrence in 1000 bootstrap replicates.

Although strangles is widely reported in horses, only a few strangles cases have been reported in donkeys with severe clinical signs. In our previous study, the strangles outbreak in donkey was first reported in intensive donkey farming (Dong *et al.*, 2019). The investigation presumed that the causative *S. equi* entered the farm with newly introduced donkeys, which suggested it is important to use quarantine measures effectively and screening methods should be used to identify the infected animals and carriers of *S. equi* to prevent the spread of strangles. Based on this information, we have been carefully monitoring other newly introduced donkeys in quarantine areas after importation. In April of 2019, one donkey showed a profuse mucopurulent nasal discharge (Fig. 1a) in the quarantine area of an intensive farm in which there were about 400 resident donkeys. The infection of *S. equi* was confirmed and diagnosed based on clinical signs, staining techniques (histochemical tests) and molecular-based techniques and was restricted to this animal. Our results emphasize the importance of quarantine areas and biosecurity to prevent outbreaks of strangles in intensive donkey farms.

It was concluded from the present study that a novel genotype of *S. equi* was identified from a donkey residing in the quarantine area of an intensive donkey farm. The increased knowledge of the molecular epidemiology of *S. equi* in populations of donkeys in China will lead to a greater understanding of the diversity of this pathogen, towards the development of effective vaccines. This report emphasizes the importance of quarantine areas and biosecurity in order to prevent incursions of *S. equi*.

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Authors contribution: WH and JD conceived the idea and designed the study. YL, NG, DH, JG, SF, YJ, TW and WZ executed the experiment. YL, NG and AW were involved in data analysis and YL and NG wrote up.

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