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

Yanlin Liu\(^1\), Nannan Gao\(^2\), Dali He\(^1\), Andrew Waller\(^3\), Jingmin Gu\(^1\), Tao Wang\(^2\), Yalu Ji\(^1\), Shushan Fan\(^2\), Dongfang Yuan\(^2\), Yan Du\(^2\), Fang Li\(^2\), Wei Zhu\(^3\), Jianbao Dong\(^2\)* and Wenyu Han\(^1,4^*\)

\(^1\)Key Laboratory of Zoonosis Research, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, China; \(^2\)Shandong Vocational Animal Science and Veterinary College, Weifang, China; \(^3\)The Animal Health Trust, Newmarket, UK; \(^4\)Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Diseases and Zoonosis, Yangzhou University, Yangzhou, China

*Corresponding author: djb922@hotmail.com; hanwy@jlu.edu.cn

**ARTICLE HISTORY**

Received: June 11, 2019
Revised: July 09, 2019
Accepted: July 10, 2019
Published online: July 30, 2019

**Key words:** Donkeys
Novel genotype SeM-138
Strangles
*Streptococcus equi*

**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.

©2019 PVJ. All rights reserved

To Cite This Article: Liu Y, Gao N, He D, Waller A, Gu J, Wang T, Ji Y, Fan S, Yuan D, Du Y, Li F, Zhu W, Dong J and Han W, 2019. Identification of a novel genotype of *Streptococcus equi* subspecies *equi* in a donkey suffering from strangles. Pak Vet J, 39(4): 609-611. http://dx.doi.org/10.29261/pakvetj/2019.087

**INTRODUCTION**

Strangles is a contagious bacterial disease of equines and is prevalent worldwide caused by host-restricted *Streptococcus equi* subspecies *equi* (Harrington et al., 2002). Clinical signs of streptococcal diseases 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 streptococcal infections were 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...
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 performed 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 outbreaks 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 widely 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.](Image 309x330 to 540x504)

![Table 1: Amino acid sequences of reference strain 4047 (SeM-3) (FM204883), SeM-88 allele, SeM-136, and SeM-138 (CN190402)](Image 309x330 to 540x504)

| Codon # | 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<sup>a</sup> | H<sup>a</sup> | -     | -       | -                 |
| 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                 |

<sup>a</sup> Insertion in SeM-88 allele.
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 strains. 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.

Acknowledgments: This work was supported by the National Key Research and Development Program of China (No. 2018YFD0500504), the National Natural Science Foundation of China (No. 31572553 and 31872505), the Jilin Province Science Foundation for Youths (Changchun, China; grant no. 20190103106JH) and the Shandong Provincial Modern Agricultural Industry Technology System (SDAIT-27).

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.

REFERENCES

Boyle AG, Timoney JF, Newton JR, et al., 2018. Streptococcus equi infections in horses: Guidelines for treatment, control and prevention of strangles-revised consensus statement. J Vet Intern Med 32:633-47.

Dong J, Gao N, Weller AS, et al., 2019. An outbreak of strangles associated with a novel genotype of Streptococcus equi subspecies equi in donkeys in China during 2018. Equine Vet J 51:743-8.

Enright MC, Spratt BG, Kalia A, et al., 2001. Multilocus sequence typing of Streptococcus pyogenes and the relationships betweenemm type and clone. Infect Immun 69:2416-27.

Harrington DJ, Sutcliffe IC and Chanter N, 2002. The molecular basis of Streptococcus equi infection and disease. Microbes Infect 4:501-10.

Kelly C, Bugg M, Robinson C, et al., 2006. Sequence variation of the SeM gene of Streptococcus equi allows discrimination of the source of strangles outbreaks. J Clin Microbiol 44:480-6.

Kumar S, Stecher G and Tamura K, 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870-4.

Meehan M, Lewis MJ, Byrne C, et al., 2009. Localization of the equine IgG-binding domain in the fibrinogen-binding protein (FgBP) of Streptococcus equi subspp. equi. Microbiology 155:2583-92.

Omer NY, Ali HAS, Harfoush RAH, et al., 2014. Molecular typing of methicillin resistant Staphylococcus aureus clinical isolates on the basis of protein A and coagulase gene polymorphisms. Int J Microbiol 2014:650328.

Slater JD, 2003. Strangles, bastard strangles, vives and glanders: archaeological relics in a genomic age. Equine Vet J 35:118-20.

van Belkum A, Tassios PT, DiJKhoorn L, et al., 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 13(Suppl 3):1-46.

Webb K, Jolley KA, Mitchell Z, et al., 2008. Development of an unambiguous and discriminatory multilocus sequence typing scheme for the Streptococcus zoöepidemicus group. Microbiology 154:3016-24.