



SHORT COMMUNICATION

Multi-Locus Sequence Typing of *Streptococcus equi* Subspecies *zooepidemicus* Strains Isolated from Donkeys: A Novel Genotype Associated with Donkey Infectious Endometritis

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ABSTRACT

Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*) is one of the most common pathogens of endometritis in intensive donkey farms. In order to investigate the molecular epidemiological characteristics of *S. zooepidemicus* isolates associated with endometritis in intensive donkey breeding farms in China, isolates (n=40) from 8 small and medium-sized breeding farms were selected for multilocus sequence typing analysis. Results revealed that 4 sequence types (STs) were identified, ST360, ST380, ST413, and a novel genotype ST-novel, and these were genetically distant from each other, which suggested that *S. zooepidemicus* strains caused endometritis in intensive donkey farms possessed high genetic diversity and were lack of genotype specificity. The result of present study contributed to the understanding of the molecular epidemiological characteristics of *S. zooepidemicus* isolates.

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INTRODUCTION

Streptococcus equi subspecies *zooepidemicus* (*S. zooepidemicus*), a beta-hemolytic Gram-positive Lancefield group C bacterium, acts as an opportunistic pathogen that has been identified in a wide range of animal species (Baracco, 2018; Azpiroz *et al.*, 2023). *S. zooepidemicus* strains have often been isolated from horses which caused mild to severe infections such as endometritis, pneumonia, adenitis, and others (Rasmussen *et al.*, 2013; Gao *et al.*, 2020; Azpiroz *et al.*, 2023). It has also been associated with various other diseases in domestic animals, encompassing respiratory infections in dogs and cats (Rasmussen *et al.*, 2013; Azpiroz *et al.*, 2023), polyarthritis in sheep (Baracco, 2018), and mastitis in cattle (Azpiroz *et al.*, 2023). In humans, it has been reported to be associated with the consumption of inadequately pasteurized dairy products and can cause serious infections such as pneumonia, endocarditis, septic arthritis, osteomyelitis, meningitis, septicemia, and myositis (Madzar *et al.*, 2015; Kerdsin *et al.*, 2022; Azpiroz *et al.*, 2023). Meanwhile, it is part of the resident

flora in the caudal reproductive tract and causes infection of the uterus or placenta during mating or in the event of tissue damage, which can lead to endometritis, uterine sepsis, early embryonic death, miscarriage, premature birth, and intrauterine growth restriction of the embryo (Gao *et al.*, 2020; Cantelmi *et al.*, 2023; Nocera *et al.*, 2023). In addition, it has posed a considerable threat to animal welfare and caused huge economic losses in the animal industry worldwide (Baracco, 2018; Cantelmi *et al.*, 2023).

Molecular epidemiological investigation can reveal the genetic evolutionary patterns and epidemiological correlations for pathogens and provide a basis for the development of effective prevention and control strategies. Recently, molecular epidemiological analysis of *S. zooepidemicus* mainly used highly variable individual loci in the colony or multivariable regions in the genome, including pulsed-field gel electrophoresis (PFGE), M protein hypervariable regions or PCR typing of the 16S-23S-rRNA gene spacer region (Lindmark *et al.*, 1999). These methods used restriction endonucleases or PCR primers to maximize variability and were limited

by unknown mechanisms of variation in anchored regions and the rapidity of variation. At present, multilocus sequence typing (MLST) has been applied to molecular epidemiological analysis for *S. zooepidemicus* isolated from animals, which involves the identification and analysis of multiple alleles by amplifying a 400-500 bp fragment of seven housekeeping genes (Webb *et al.*, 2008; Cantelmi *et al.*, 2023; Nocera *et al.*, 2023). Specifically, this study is intended to analyze the genetic evolution of *S. zooepidemicus* isolates by using the MLST method, which leads to a great understanding of the molecular epidemiological characteristics of *S. zooepidemicus* isolates associated with endometritis in intensive donkey breeding farms in China.

MATERIALS AND METHODS

Sample collection and bacteria isolation: Ethical approval for this study was granted by the Ethics Committee at the Shandong Vocational Animal Science and Veterinary College. The genital swab samples and purulent vaginal discharge samples were collected from female donkeys in 8 small or medium-sized breeding farms, as described previously (Gao *et al.*, 2020). A total of 40 isolates of *S. zooepidemicus* were included in this study. All isolates were cultured on blood agar and stored long-term in brain heart infusion (BHI) broth with 30% glycerol at -80°C . Each isolate was cultured overnight at 37°C and bacterial genomic DNA was extracted with a bacteria DNA kit (TIANamp, Beijing, China) according to the manufacturer's instructions.

MLST: In MLST assays, seven housekeeping genes, i.e., carbamate kinase (*arcC*), ribonucleoside-diphosphate reductase (*nrpE*), propyl-tRNA synthetase (*proS*), signal peptidase I (*spi*), thymidylate kinase (*tdk*), triosephosphate isomerase (*tpi*) and acetyl-CoA acetyltransferase (*yqiL*) were amplified by PCR as described previously (Webb *et al.*, 2008). Thermal cycling conditions for PCR were 94°C for 2min; 30 cycles of 94°C for 20s, 55°C for 30s, and 72°C for 30s; a final elongation step of 72°C for 5min. All PCR primers used in this study are described by Webb *et al.* (2008) and sequencing was performed by Sangon Biotech.

ST analysis and clustering tree construction: The MLST sequence data from Sangon Biotech (Shanghai, China) was assembled and analyzed using the *S. zooepidemicus* MLST scheme as described previously (Webb *et al.*, 2008). The results were uploaded to the PubMLST *S. zooepidemicus* database (<https://pubmlst.org/organisms/streptococcus-zooepidemicus/>) for sequence type analysis and clustering tree of ST of *S. zooepidemicus* isolates was constructed by Phyloviz 2.0 software.

RESULTS AND DISCUSSION

MLST analysis is one of the most powerful tools for genotyping specific bacterial species based on multiple internal fragments of housekeeping genes amplified by PCR (Webb *et al.*, 2008; Cantelmi *et al.*, 2023; Nocera *et al.*, 2023). MLST has been considered an easy-using and

higher-resolution molecular analysis method and can classify the same bacteria species into more subtypes and help to identify the phylogenetic relationships between different subtypes and their relevance to disease (Webb *et al.*, 2008). Since 2008, MLST has been successfully applied to the genotyping and molecular epidemiological analysis for *S. zooepidemicus* (Webb *et al.*, 2008). In the present study, a total of 4 STs were identified in 40 *S. zooepidemicus* isolates collected from female donkeys in 8 small or medium-sized breeding farms. Three of these STs had already registered as ST360, ST380 and ST413 in the MLST database, whereas one was identified as a novel genotype by the database and was tentatively named as ST-novel in this study (Fig. 1). Allelic profile of housekeeping genes and STs of *S. zooepidemicus* isolates were shown in Table 1, which showed farms SD4 and SD7 possessed 2 STs of ST360 and ST380, ST360 and ST413, respectively. While other farms only had one ST. For the limitation of the present study, there were only 5 strains selected for MLST analysis from each farm, which might not accurately identify all genotypes presented in the farm due to the limited number of strains. Therefore, more strains are needed to be selected for analysis in further study.

Infectious endometritis is one of the most common infectious diseases in intensive donkey breeding farms in China, and *S. zooepidemicus* isolates were found to be one of the major causative pathogens (Gao *et al.*, 2020). In order to understand the molecular epidemiological characteristics of *S. zooepidemicus* isolates associated with endometritis in intensive donkey farms, the evolutionary analysis was performed, and an ST clustering tree was constructed for 4 STs identified in the present study and others in the PubMLST database of *S. zooepidemicus*. It illustrated these 4 STs were widely dispersed in the phylogenetic tree, and they kept a long genetic distance from each other (Fig. 1), which indicated that the isolates of *S. zooepidemicus* possessed highly genetic diversity in cases of endometritis in intensive donkey farms. Moreover, it suggested there might be many genotypes of *S. zooepidemicus* in intensive donkey farms, and they possessed high genetic diversity in cases of endometritis. This might be attributable to the fact that *S. zooepidemicus* is an opportunistic pathogen and persists in living on the body surface of animals and the surrounding environment (Azpiroz *et al.*, 2023). Since it has strong vitality and can live for a long time in intensive donkey farms, many strains of *S. zooepidemicus* can live independently from each other and are evaluated constantly, and then forms many genotypes in farms under the hypothesis.

As described in PubMLST database of *S. zooepidemicus* and shown in the STs clustering tree (Fig. 1), the representative strain of ST360 was isolated from horses in New Zealand in 2015 and was derived from ST355 and ST323 which representative strains were identified in horses in Iceland in 2013. As well as ST380 was also derived from ST435, which representative strain CE785 was associated with uterine infection and abortions in horses in Argentina in 2018. Meanwhile, ST413 was derived from ST174, whose representative strains 3022 and WK17089_1 was associated with pneumonia and guttural pouch infection in the United States and New

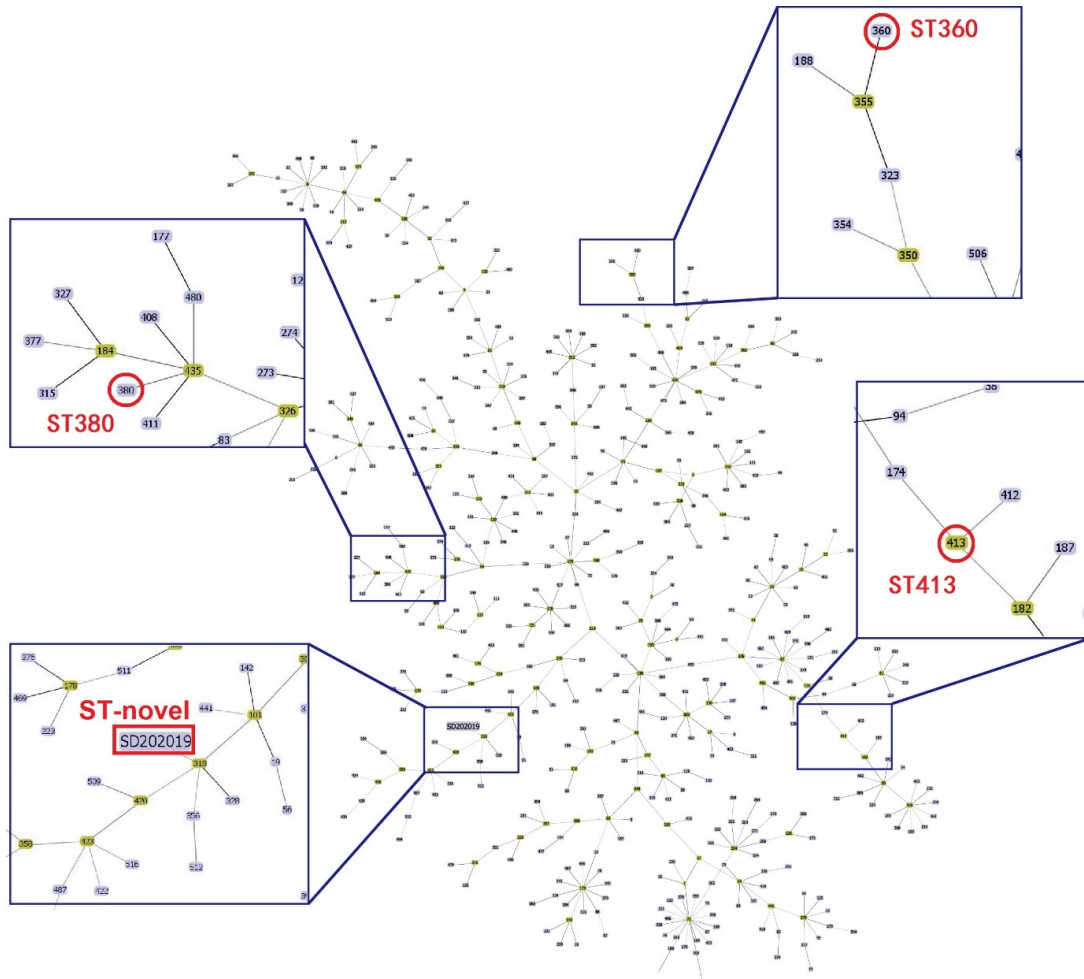


Fig. 1: Clustering tree of ST of *S. zooepidemicus* isolates. The clustering tree was constructed based on the analysis of 7 house-keeping genes *arcC*, *nrdE*, *proS*, *spi*, *tdk*, *tpi* and *yqjL* by Phyloziz 2.0 software.

Table 1: Allelic profile of house-keeping genes and ST types of *S. zooepidemicus* isolates

ST type	<i>arcC</i>	<i>nrdE</i>	<i>proS</i>	<i>spi</i>	<i>tdk</i>	<i>tpi</i>	<i>yqjL</i>	Source (breeding farm)
ST360	39	13	15	34	25	31	44	SD3, SD4, SD7
ST380	31	3	4	35	1	23	67	SD4, SD6
ST413	4	12	7	6	11	7	6	SD1, SD2, SD7, HNI
ST-novel	12	10	45	24	14	6	novel	SD5

Zealand, respectively. Moreover, ST412 was one of the derived from ST413 and its representative strain CE782 was associated with uterine infection and abortions in horses in Argentina in 2018. As described above, ST360, ST380, and ST413 were all derived from the genotypes of equine origin.

Furthermore, the novel genotype ST-novel was derived from ST318, whose representative strain HT321 was a canine isolate identified in the United States in 2008 but not of equine origin. However, ST420 was also derived from ST318 and was isolated from donkey strangle cases in Xinjiang Province, China in 2020. It suggested that the genotype of *S. zooepidemicus* was not associated with host specificity, and there was no genotype specificity for *S. zooepidemicus* infection between the reproductive and respiratory system.

Conclusions: MLST was performed for *S. zooepidemicus* isolates associated with endometritis in intensive donkey farms. A total of 4 STs, including one novel genotype,

were identified by MLST analysis. They were widely dispersed in the clustering tree and kept a long genetic distance from each other, which indicated that the isolates of *S. zooepidemicus* possessed highly genetic diversity in cases of endometritis in intensive donkey farms.

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Author's contribution: JD conceived the idea and designed the study. NG, CC, YJ, JG, YD, WZ, JZ, and JD executed the experiment. NG, CC, and YJ were involved in data analysis and writing.

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