The Journal of Veterinary Medical Science

NOTE

Bacteriology

Genetic analysis of *Streptococcus equi* subsp. *equi* isolated from horses imported into Japan

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J. Vet. Med. Sci.
81(6): 924–927, 2019
doi: 10.1292/jvms.18-0656

Received: 7 November 2018
Accepted: 11 April 2019
Published online in J-STAGE: 24 April 2019

ABSTRACT. Strangles is a commonly diagnosed and important infectious disease of equids worldwide, caused by *Streptococcus equi* subsp. *equi*. We determined the SeM genotypes of *S. equi* isolated from imported horses at the Japanese border within the past 8 years, which allowed us to classify 12 strains isolated from these horses from each exporter into four allelic groups. These alleles were different from the alleles of past isolates found in Japan. Furthermore, four strains classified into the same allele were isolated from horses from one exporter over several years. In this study, *S. equi* isolates from different exporters had different SeM alleles. Attention to the hygiene status of farms will be necessary to prevent the incursion of strangles.

KEY WORDS: Animal Quarantine Service, genetic analysis, SeM, *Streptococcus equi*

Strangles is a commonly diagnosed and important infectious disease of equids worldwide, caused by *Streptococcus equi* subsp. *equi* [7, 14, 16]. It is characterized by a mucopurulent nasal discharge and acute swelling with subsequent abscess formation in the submandibular and retropharyngeal lymph nodes [4, 14, 16]. *S. equi* infects the horse's cranial lymph glands and is highly communicable to other horses. Outbreaks of strangles have been reported in many countries, and sporadic outbreaks have occurred in Japan since 1992 [2, 5, 7, 18]. In recent years, the number of draft horses imported into Japan for a feeder has increased, and accounts for approximately 85% of the total volume of imported horses; strangles has occurred only in herds of draft horses during the quarantine period of importation. In the past, the outbreak of strangles in domestic horses was thought to be caused by imported draft horses [2]. Molecular studies have used the gene sequence encoding the N-terminal end of the M-like protein (SeM) for genotyping and identifying the sources of the outbreaks [1, 6, 8–11]. The purpose of this study was to investigate the genetic relationships among *S. equi* strains isolated from imported draft horses within the past 8 years at the Japanese border by the Animal Quarantine Service (AQS) using SeM genotyping, to confirm colonial variants of the strains, and to determine minimum inhibitory concentration (MIC) of antibiotics against the strains for evaluating the treatment and control strategies.

Twelve *S. equi* isolates were collected from imported draft horses from 2010 to 2017 (Table 1). Bacterial isolation from the nasal swab was performed. Swabs were streaked on Columbia agar plate (Columbia Agar; Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) with 5% sheep blood and incubated at 37°C for 24 to 48 hr under 5% CO_2 conditions. Two of the 12 isolates were isolated in the AQS Moji branch and 10 were isolated at Kagoshima Airport, Moji sub-branch. Eleven isolates were isolated from clinical cases in which the horses showed fever, nasal discharge, an increase in the white blood cell count, and/or swelling of the submandibular lymph nodes (all except No. 11). The horses were imported by 5 Canadian exporters.

The isolates were cultured on Columbia agar with 5% sheep blood at 37°C for 24 hr. After the incubation, genomic DNA was extracted from the bacterial colony using a DNA extraction kit (InstaGene Matrix; Bio-Rad Laboratories, Hercules, CA, U.S.A.). The isolates were cultured for more than 24 hr to observe the colony morphology.

The entire structural SeM gene was amplified by PCR using the primer pairs; 5′-CAA AAA AGT GTG CCC ATA AC-3′ and

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Table 1. Characterization of *Streptococcus equi* isolates in this study

Isolate No.	SeM allele	Exporter	Year of isolation	Clinical or subclinical	Minimum inhibitory concentration of antibiotics[a)]							
					ABPC	CEZ	CTX	KM	GM	TC	CP	CPFX
1	SeM-39	B	2010	Clinical	≤1	≤1	≤0.5	16	2	≤0.5	2	1
2	SeM-43	A	2010	Clinical	≤1	≤1	≤0.5	8	≤0.5	≤0.5	2	1
3	SeM-43	A	2010	Clinical	≤1	≤1	≤0.5	16	2	≤0.5	4	1
4	SeM-28	D	2011	Clinical	≤1	≤1	≤0.5	4	2	≤0.5	4	1
5	SeM-28	D	2011	Clinical	≤1	≤1	≤0.5	8	2	≤0.5	2	1
6	SeM-39	B	2013	Clinical	≤1	≤1	≤0.5	8	2	≤0.5	4	1
7	SeM-30	C	2015	Clinical	≤1	≤1	≤0.5	16	2	1	2	1
8	SeM-30	C	2015	Clinical	≤1	≤1	≤0.5	8	1	≤0.5	2	1
9	SeM-28	E	2011	Clinical	≤1	≤1	≤0.5	16	1	≤0.5	2	1
10	SeM-28	E	2011	Clinical	≤1	≤1	≤0.5	2	≤0.5	≤0.5	2	1
11	SeM-39	B	2017	Subclinical	≤1	≤1	≤0.5	4	1	≤0.5	2	2
12	SeM-39	B	2017	Clinical	≤1	≤1	≤0.5	16	1	≤0.5	2	1

a) Antibiotics: ABPC=ampicillin, CEZ=cefazolin, CTX=cefotaxime, KM=kanamycin, GM=gentamycin, TC=tetracycline, CP=chloramphenicol, CPFX=ciprofloxacin.

5′-TCG AAG TTG GGA ATC TCT-3′ [1], and 5′-CAG AAA ACT AAG TGC CGG TG-3′ and 5′-ATT CGG TAA GAG CTT GAC GC-3′ [7]. The PCR was performed in a final volume of 50 μl containing 5 μl of 10× *Ex Taq* buffer, 4 μl of 2.5 mM of each deoxynucleotide triphosphate (dNTP) mixture, 1 μl of 20 μM of each primer, 0.25 μl of 5 units/μl *Ex Taq* (*TaKaRa Ex Taq*; Takara Bio Inc., Kusatsu, Japan), 1 μl of DNA template, and distilled water. Amplification was performed using an initial denaturation for 2 min at 94°C, followed by 25 cycles of 2 min at 95°C, 1 min at 55°C and 2.5 min at 72°C. After amplification, the PCR products were electrophoresed on 2% agarose gel in Tris-acetate-EDTA buffer. The PCR products were purified and concentrated using a purification kit (Illustra GFX PCR DNA Purification Kit; GE Healthcare, Piscataway, NJ, U.S.A.). The nucleotide sequences were determined using a commercial kit (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) and a DNA sequencer (3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific) in accordance with the manufacturer's protocol.

In the determined nucleotide sequences, 327 bases of each strain were used for SeM allele typing and 492 bases were used for phylogenetic analysis. SeM alleles were identified by comparison of gene sequence data with the SeM database (http://pubmlst.org/szooepidemicus/seM). Sequencing data were analyzed using software (GENETYX Network version 12.0.1; GENETYX Corp., Tokyo, Japan) and phylogenetic analysis was carried out using neighbor-joining trees with 1,000 replicates in a bootstrap analysis on CLUSTAL W [15]. GENETYX-Tree (GENETYX) was used to display the phylogenetic tree.

MIC of antibiotics against the 12 strains was determined using the broth micro-dilution technique (Dry plate; Eiken Chemical Co., Ltd., Tokyo, Japan). The antibiotics used were ampicillin, cefazolin, cefotaxime, kanamycin, gentamycin, tetracycline, chloramphenicol, and ciprofloxacin. We determined the antimicrobial susceptibility and resistance of the strains under the MIC breakpoints per CLSI standards.

Analysis of the SeM sequence data resulted in the classification of 12 strains of *S. equi* into 4 allelic groups (alleles 28, 30, 39, and 43). Two strains were isolated from horses from exporter A and had allele 43, 4 were isolated from horses from exporter B and had allele 39, 2 were isolated from horses from exporter C and had allele 30, and 4 were isolated from horses from exporters D and E and had allele 28 (Table 1). These 4 alleles were different from alleles identified in past isolates in Japan (alleles 18, 19 and 20) (Fig. 1). The four isolates from horses from exporter B (allele 39) were obtained in 2010, 2013, and 2017 (Fig. 1). All 12 strains formed matt-type colonies (Fig. 2) and were susceptible to beta-lactam antimicrobials (Table 1).

The 12 strains identified in this study were classified into 4 SeM allelic groups. One of them (Allele 28) was the same allele as that in a strain isolated in the U.S. in 1998 [1], but all four alleles were different from those in past strains isolated from domestic race and riding horses in Japan [2, 18]. This indicates that draft horses imported recently from Canada may have little potential to spread strangles to domestic horses. In addition, domestic horses are also protected from invasion of strangles from imported draft horses by the policy of properly separating the imported horses from domestic ones after quarantine inspection.

The molecular phylogenetic tree analysis revealed that the four strains isolated from the herds of exporter B in 2010, 2013, and 2017 were closely related and had the same allele (allele 39). This suggests that *S. equi* had been present in the farms of exporter B for a long time. Each cluster of stains in this study corresponded to one allele and was found in horses from 1 to 2 exporters. This suggests that the strains of *S. equi* in Canada have some SeM alleles. Furthermore, by continuing the investigation using this method at Japan's border, it might be possible to determine whether the farms used by the exporters contain horses that have strangles. This would allow us to provide more useful information regarding preventive measures for strangles to importers of draft horses. In this study, only the information described in the health certificate issued by the government agency of the exporting country was available to us. We require more information, including information on the farms of origin, movement of horses, and vaccination against strangles for more detailed studies.

It is known that there are colonial variants of *S. equi* [12, 13, 17] and the anti-phagocytic effects of the hyaluronic acid capsule

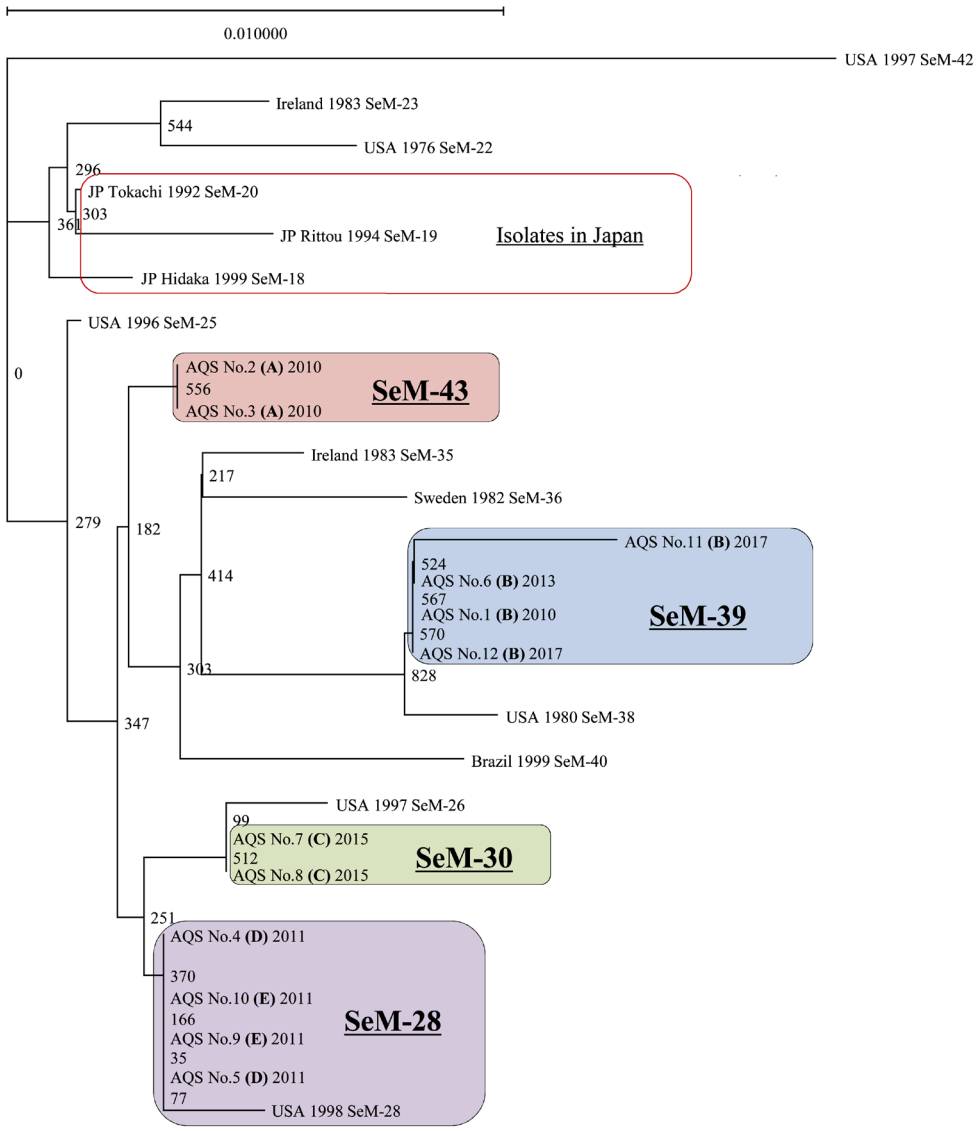

Fig. 1. Neighbor-joining phylogenetic tree constructed on the basis of the nucleotide sequences of SeM alleles. Bootstrap values are shown in the tree branches (1,000 replicates were performed). Letter in parentheses is the marks of exporters.

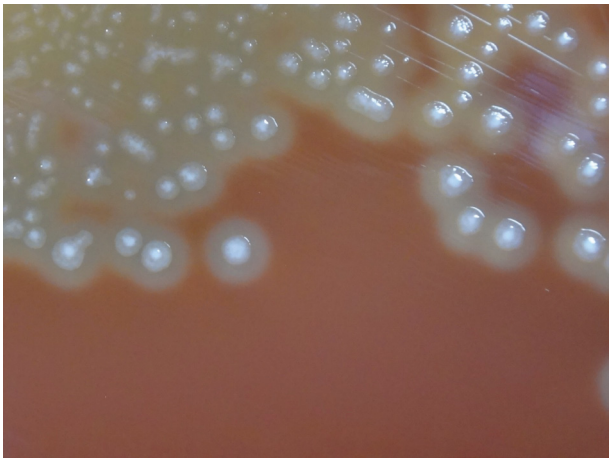

Fig. 2. *Streptococcus equi* showing matt-type colony on Columbia agar with 5% sheep blood at 37°C for 48 hr.

on the pathogenicity in horses have been evaluated [3]. Mucoid colonies express large capsules and have been isolated from cases of classical strangles. Matt strains are lysogenized with bacteriophages encoding hyaluronidase, which digests the capsule within 8–10 hr [13, 17], and have been associated with mild atypical strangles [12]. In foreign countries, approximately 80% of *S. equi* isolates form mucoid colonies, but all strains isolated from imported horses in this study formed matt-type colonies. This suggests that there were subclinical carriers or horses with clinically mild onset of strangles due to matt-type *S. equi* in the herds. These horses then contracted typical strangles after arriving in Japan due to the stress associated with the long duration of air transportation.

In general, *S. equi* is susceptible to beta-lactam antimicrobials, such as penicillin and cephem antibiotics, which have been used to treat strangles [14]. None of the strains isolated from imported horses in this study had antimicrobial resistance to these antibiotics. Therefore, the above antibiotics should be effective for the treatment of horses with strangles imported from Canada.

In conclusion, the SeM alleles of strains isolated from imported draft horses were different from those found in domestic race and riding horses in Japan. SeM sequencing analysis revealed that the *S. equi* isolates from each exporter had different SeM alleles. Continuous investigations using this method at Japan's border might enable us to evaluate the hygiene status of the farms of the exporters. More attention will be necessary to prevent the incursion of strangles via imported horses in the future.

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