Variation in the N-terminal region of an M-like protein of *Streptococcus equi* and evaluation of its potential as a tool in epidemiologic studies

Toru Anzai, DVM, PhD; Yasushi Kuwamoto, DVM; Ryuichi Wada, DVM, PhD; Sigeo Sugita, PhD; Tsutomu Kakuda, DVM, PhD; Shinji Takai, DVM, PhD; Tohru Higuchi, DVM, PhD; John F. Timoney, MVB, PhD, DSc

Objective—To develop a method for typing *Streptococcus equi* on the basis of the DNA sequence of the genes that produce an M-like protein and to compare isolates among the United States, Japan, and other countries.

Sample Population—*S equi* strains CF32, Hidaka/95/2, and NCTC9682 as well as 82 other isolates from the United States, Japan, and other countries obtained during 1975 to 2001.

Procedure—DNA sequences of the structural genes (*SeM* and *SzPSe*) that produce M-like proteins were determined for 3 representative strains to find a variable region. Variability in this region of *SeM* was then determined for the other isolates. Amino acid sequences were deduced and analyzed phylogenetically by use of the neighbor-joining method.

Results—Sequence diversity was detected in the N-terminal region of *SeM* but not in *SzPSe* of the 3 representative strains. Base substitutions in the variable region of *SeM* varied in a nonsynonymous manner, resulting in variation in the amino acid sequence. Eighty-five isolates were categorized as 32 types of *SeM* on the basis of differences in the deduced amino acid sequences.

Conclusions and Clinical Relevance—This study documented a region in the N-terminal portion of *SeM* that varies in a nonsynonymous manner. This information should be useful in molecular epidemiologic studies of *S equi*. (*Am J Vet Res* 2005; 66:2167–2171)

Streptococcus equi (*S equi* subsp *equi*), a Lancefield group C streptococcus, causes strangles, an important contagious disease of equids throughout the world. *Streptococcus equi* has almost no biological, biochemical, serologic, or genetic variation,[1-4] although phenotypic variations in colony type attributable to differences in capsule expression of hyaluronic acid have been reported.[5] There have been few epidemiologic studies based on differences among *S equi* isolates. Large restriction fragment polymorphism by use of pulse-field gel electrophoresis and repetitive sequence–based **polymerase chain reaction (PCR)** assays have been used as methods for molecular typing of *S equi*.[6,7] A disadvantage of these techniques is that they are based on comparisons of patterns of electrophoretic bands; thus, it is necessary to use standard or reference strains for the typing of field isolates. A method based on the DNA sequence in a specific gene does not require reference strains because sequence data are already known.

The cell wall–associated protein (SeM protein) is a major virulence factor and protective antigen of *S equi*.[8] This protein inhibits deposition of the complement component C3b on the surface of bacteria,[9] binds equine fibrinogen and IgG-Fc, and enhances resistance to phagocytosis.[10-12] Because SzPSe is a homologue of the M-like protein SzP produced by *Streptococcus zooepidemicus* (*S equi* subsp *zooepidemicus*),[8,13] it is an antigen that can be used for typing. This protein is of unknown function in *S equi* but is an important protective antigen in *S zooepidemicus*. The DNA sequences of these M-like proteins were originally obtained from the US prototype strain CF32. The DNA sequence of the *SeM* gene was subsequently confirmed in the European isolate TW.[10]

The objectives of the study reported here were to determine the sequence variability of the M-like proteins of *S equi* and develop a molecular method for typing *S equi* on the basis of these differences. Another objective was to validate the method by use of *S equi* isolates collected in the United States, Japan, and other countries.

Materials and Methods

Sample population—Strains CF32, Hidaka/95/2, and NCTC9682, which form mucoid, matt, and glossy colonies, respectively, were used as prototypic representative strains of *S equi*.[5] Strain CF32 was isolated in New York in 1981, strain Hidaka/95/2 was isolated in Japan in 1995,[14] and strain NCTC9682 was isolated in Europe in the 1950s. Eighty-two isolates from horses with strangles in the United States, Japan, and other countries were obtained during the period from 1945 to 2001 (**Appendix**). Twenty-five isolates from Japan were obtained during outbreaks of strangles in horses on 12 farms. These isolates were identified by use of a slide-agglutination technique for Lancefield group C,[a] a commercially available kit for the

Received December 1, 2004.
Accepted March 10, 2005.
From the Epizootic Research Center, Equine Research Institute, Japan Racing Association, 1400-4 Shiba, Kokubunji-machi, Shimotsuga-gun, Tochigi 329-0412, Japan (Anzai, Kuwamoto, Wada, Sugita); the Department of Animal Hygiene, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034-8628, Japan (Kakuda, Takai); the Mitsuishi Animal Clinic Center, Agriculture Mutual Aid Association of Hidaka District 200 Higashihohrai, Mitsuishi-cho, Mitsuishi-gun, Hokkaido 059-3105, Japan (Higuchi); and the Gluck Equine Research Center, Department of Veterinary Science, College of Agriculture, University of Kentucky, Lexington, KY 40546-0099 (Timoney).
The authors thank Junko Goma and Humiko Yomogida for technical assistance.
Address correspondence to Dr. Anzai.

detection of streptococci,[b] and immunoblotting with antisera specific for M-like proteins.[2]

DNA preparation—A colony of each strain or isolate cultured initially on Columbia agar[c] with 5% horse blood was subcultured into 1 mL of Todd-Hewitt broth[d] with 0.2% yeast extract and 10 units of hyaluronidase. After incubation at 37°C for 18 hours, the subculture was centrifuged at 15,000 × *g* for 1 minute and supernatant was harvested. Two hundred microliters of matrix[e] for preparation of DNA for the PCR template was added to the bacterial cell pellet, and genomic DNA was extracted in accordance with the manufacturer's instructions.

DNA sequencing—The entire structural genes of *SeM* and *SzPSe* from strains CF32, NCTC 9682, and Hidaka/95/2 were sequenced from amplicons by use of PCR primers designed from the sequence[8] of CF32 and by use of the dideoxy-chain termination method with a genetic analyzer.[f] The variable region of the *SeM* gene was sequenced by use of a primer pair (5'–CAA AAA AGT GTG CCC ATA AC–3' and 5'–TCG AAG TTG GGA ATC TCT–3') to amplify the N-terminal portion of the structural gene (from nucleotides 18 to 492) and the deduced amino acid sequence. The PCR assay was performed by use of 25 cycles (each cycle consisted of 95°C for 2 minutes, 55°C for 1 minute, and 72°C for 2.5 minutes).

Phylogenic tree analysis—Phylogenetic analysis was performed by use of a molecular, evolutionary analysis system.[15,g] Sequences were initially aligned by use of a multiple-sequence alignment method.[16] Robustness of the grouping was tested by use of bootstrap resampling. The neighbor-joining tree with 500 replications was used in the bootstrap analysis.[17]

Results

Strains CF32, Hidaka/95/2, and NCTC9682 were subcultured on blood agar 10 times, and the DNA sequences of genes *SeM* and *SzPSe* in the original and subcultured bacteria were determined twice. We did not detect any changes in DNA sequence. The DNA sequence of *SeM* gene of *S equi* CF32 comprised 1,605 nucleotides, as reported elsewhere.[8] However, 7 nucleotides differed from the published sequence. These differences were at positions 23 (C to A); 525 (G to A); 537 (G to A); 540, 541, and 542 (CAC to ACA); and 1569 (A to C). The *SeM* genes of Hidaka/95/2 and NCTC9682 also contained 1,605 nucleotides. Four nucleotides in Hidaka/95/2 (position 112, C; position 174, A; position 187, A; and position 319, G) differed from the sequence of CF32 (position 112, T; position 174, T; position 187, G; and position 319, A). For NCTC9682, nucleotides at positions 174, 187, and 429 were G, A, and T, respectively. The *SzPSe* gene of CF32 consisted of 1,122 nucleotides, as described elsewhere,[8] but it differed at 4 positions from the published sequence (positions 88 and 89, CG to GC; position

Table 1—Differences in the amino acid sequence of the N-terminus among *SeM* gene types for isolates of *Streptococcus equi* analyzed by use of a multiple sequence alignment method.[16]

Type	8	38	47	51	52	53	54	56	57	58	62	63	64	65	69	99	102	103	106	107	108	110	111	113	122	125	127	143
1*	Q	S	R	D	L	K	N	L	S	D	S	G	D	A	Q	R	Y	Y	L	M	H	S	S	L	R	S	A	R
2	–	P	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–
3	–	P	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–
4	–	P	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	P	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–
6	–	P	–	–	–	–	–	–	–	E	–	–	–	–	–	–	–	–	–	K	–	P	–	–	–	–	–	–
7	–	P	–	–	–	–	–	–	–	E	–	–	–	–	–	–	–	H	–	–	–	–	–	–	–	–	–	–
8	–	P	–	–	–	–	–	–	–	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–	–	R	–	L	–	–	–	–	–
11	–	–	–	–	–	–	–	–	–	–	–	R	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	–	–	–	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–
14	–	–	–	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	S	–	R	–	–	–	–	–	–	–
15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	F	–	–	–	–	–	–	–	–	–	–	–
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	F	H	–	–	R	–	–	–	–	–	–	–
17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	P	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		S
20	–	–	–	–	–	–	–	–	–	–	D	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	S
21	–	–	–	–	–	–	–	–	–	–	–	R	–	–	L	–	–	–	–	–	–	–	–	–	–	–	–	S
22	–	–	–	–	–	–	S	–	N	E	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S
23	–	–	–	–	–	–	–	–	N	–	–	R	–	–	–	K	–	–	–	–	–	P	–	–	–	–	–	S
24	–	–	–	–	–	–	–	–	N	–	–	R	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	S
25	–	–	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S
26	–	–	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	–	–	–	–	S	N	–	S
27	–	–	–	–	F	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	–	–	–	S	–	N	–	S
28	–	–	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S
29	K	–	–	–	–	–	–	–	–	E	–	R	–	–	–	–	–	–	–	–	Q	–	–	–	–	–	–	S
30	–	–	K	N	–	R		F	–	E	–	R	–	–	–	–	–	N	–	–	R	P	P	–	S	–	–	–
31†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
32‡	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Numbers at the top of the table represent the position of the amino acid from the N-terminus of *S equi* CF32. Amino acid sequences (residues 1 to 164) were compared with those for CF32.

*Strain CF32 was designated as type 1. †AQKAQK were inserted between residues 68 and 69. ‡AQK were inserted between residues 68 and 69.

Q = Glutamine. S = Serine. R = Arginine. D = Aspartic acid. L = Leucine. K = Lysine. N = Asparagine. G = Glycine. A = Alanine. Y = Tyrosine. M = Methionine. H = Histidine. P = Proline. E = Glutamic acid. V = Valine. T = Threonine. F = Phenylalanine.

– = Same amino acid sequence as for type 1.

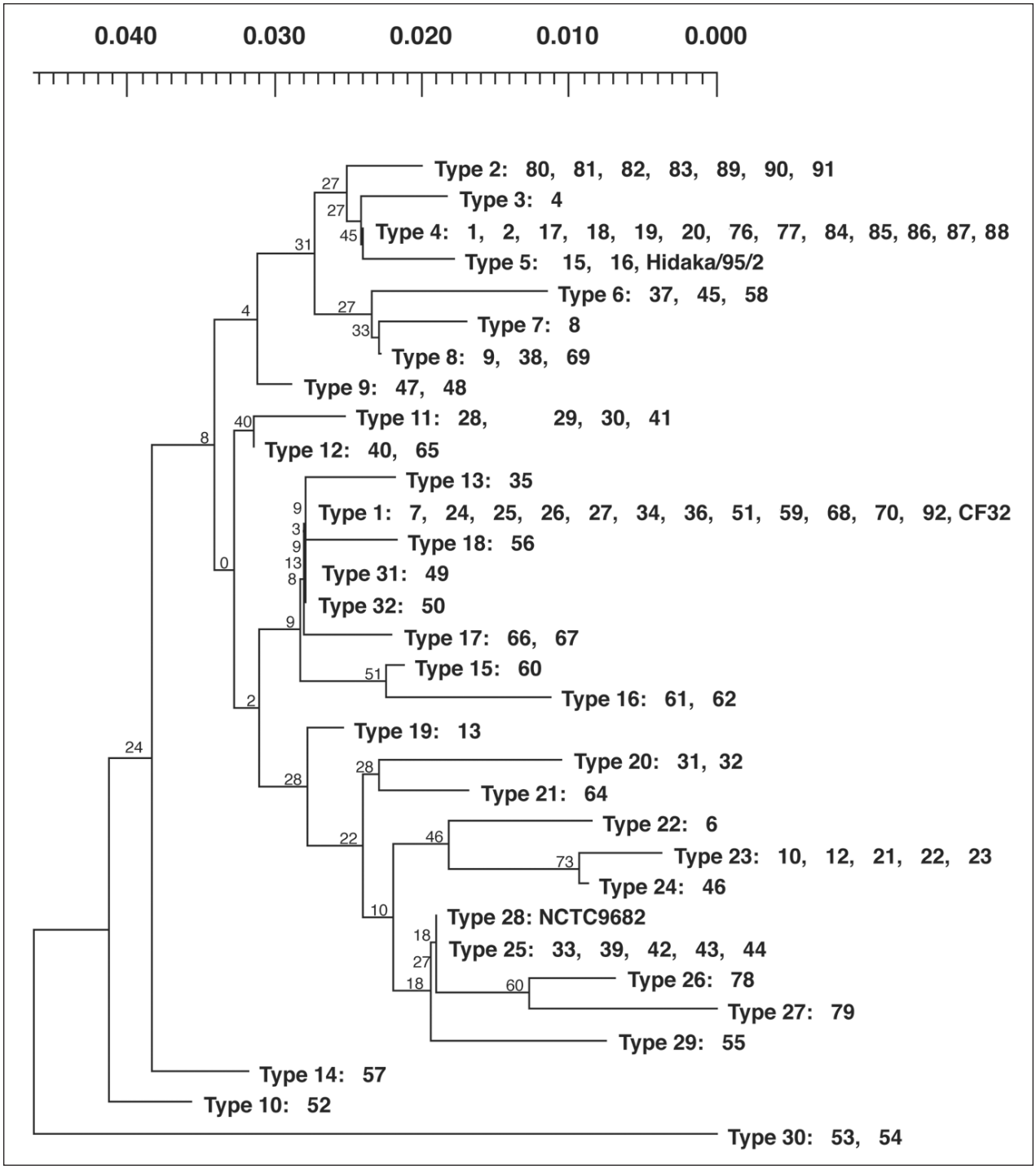

Figure 1—Phylogenic tree for 85 *Streptococcus equi* isolates based on the sequence of amino acids in the N-terminus (amino acids 1 to 164) of the *SeM* gene. The tree was constructed by use of the neighbor-joining method and 500 replications for a bootstrap analysis. The *SeM* type and identification number of isolates are indicated for the various branches. Numbers at the left side of each branch represent the bootstrap probability/100 trials. The scale at the top represents the molecular evolutionary distance per site per year.

635, G to A; and position 797, T to C). The *SzPSe* genes of Hidaka/95/2 and NCTC9682 were identical to those of CF32.

The N-terminal nucleotide sequences of the variable region of the *SeM* gene in 82 isolates were determined up to position 492. Variations were found in 70 of 82 isolates. Base substitutions were nonsynonymous, except for single bases in isolates 31 and 32 (position 312, C). On the basis of analysis of the amino acid sequence deduced from the nucleotide sequence, 32 *SeM* gene types were detected among the 85 isolates. Amino acid substitutions at 28 positions, compared with the amino acid sequence for the *SeM* gene of *S equi* CF32, were detected (**Table 1**).

A phylogenetic analysis of *SeM* gene types was conducted on the basis of the deduced amino acid sequences (**Figure 1**). The *SeM* type 1 included strain CF32 isolated in New York in 1981; bacteria isolated in New York, Kentucky, and Minnesota after 1981; and isolate No. 92 cultured in Japan in 2001. Type 1 was closely related to types 13, 15, 16, 17, 18, 31, and 32, all of which were isolated in the same geographic region and during the same time period. All isolates from Japan, except isolate No. 92, formed a cluster composed of *SeM* types 2, 3, 4, and 5. The isolates cultured during an outbreak of strangles were of the same *SeM* gene type. These major *SeM* types from Japan were closely related to *SeM* types 6 and 8 that comprised isolates cultured in New York and Ohio before 1980 and *SeM* type 7 that was isolated in Ireland in 1983. Strain NCTC9682 was *SeM* type 28 and related to *SeM* types 19, 20, 21, 22, 23, 24, 25, 26, 27, and 29. This group included isolates from countries other than the United States and Japan.

Discussion

In another study,[2] investigators used immunoblot analysis to document that the molecular weights of genes *SeM* and *SzPSe* of *S equi* isolated in various places and at various times are similar. Results of the study reported here, which were determined on the basis of gene analysis, supported that conclusion. The *SzPSe* gene was identical in strains CF32, Hidaka/95/2, and NCTC9682. Although the number of amino acids encoded by the *SeM* structural gene is the same for these 3 strains, amino acid substitutions were evident in a variable region for a small number of isolates. Examination of partial sequences of the variable region in 82 isolates revealed that the number of amino acids was unchanged in all isolates except isolates 49 and 50.

Interestingly, synonymous variations were found in the *SeM* gene of only 1 of 85 isolates, yet nonsynonymous variations were evident in a number of codons in the N-terminal portion of the *SeM* gene. For most proteins, the mean rate of synonymous variation exceeds that of nonsynonymous variation by a factor of 5.4 because the latter are likely to impair functional fitness.[16] Thus, the N-terminal domain must have a less tight functional constraint than the central or carboxy-terminal regions. This may be explained by conformational requirements. Synonymous mutations will occur at about half the rate of nonsynonymous mutations when the affected domain is nonfunctional.[16] Change of an amino acid in the *SeM* gene may change an epitope and therefore affect bacterial survival in the host. It has been suggested[11,18] that the noncoiled-coil N-terminus of the *SeM* gene is involved in fibrinogen binding and that the flanking coiled-coil stabilizes this interaction. Furthermore, those investigators proposed that the coiled-coil structures of the *SeM* gene and fibrinogen interact in a zipperlike manner.[11,18] The observed variations in amino acids of the *SeM* gene indirectly support their theory that this region has an important role in the function of *S equi*.

Typing methods based on variation of nucleotide sequence or the sequence of amino acids are superior to other molecular methods. The amino acid sequence of the variable region of the *SeM* gene rarely changes as determined on the basis of our observed lack of change during repeated subculture. Furthermore, rarity of change in the *SeM* gene type was supported by the observed lack of variation in sequences of bacteria isolated during a specific outbreak of strangles. Therefore, analysis of these observations indicates that the novel molecular typing method is universally applicable, reliable, and repeatable. In general, it is not clear when and where amino acids change. Prolonged exposure to antibodies, such as in horses with chronic infections of the diverticulum of the auditory tube (ie, guttural pouch), can result in deletions in the N-terminus of the *SeM* gene.[19] The N-terminus of the *SeM* gene contains several epitopes that react with serum and mucosal antibodies of convalescent horses and theoretically could be subject to antigenic drift.[20] However, the central area of the molecule has a greater density of epitopes but has much less variation in amino acids than does the N-terminus.

In the study reported here, 85 isolates of *S equi* were classified into 32 *SeM* types (Table 1). Isolates from Japan consisted of 5 *SeM* types, 4 of which (types 2, 3, 4, and 5) were closely related (Figure 1). These major *SeM* types in Japan were related to the cluster of US isolates from 1975 to 1980 (types 6 and 8). The phylogenetic tree analysis of major *S equi* strains in Japan supports results of another study[14] in which investigators concluded that *S equi* was transmitted to Tokachi, Japan, by horses obtained from Indiana in 1992 and then subsequently spread to other areas.

Another isolate (*S equi* 92) entered Japan through Quarter Horses obtained from the United States in 2001.[21] Its *SeM* gene type differed from that of other isolates in Japan but was similar or identical to isolates in the United States from 1981 to 1997 (types 1, 13, 15, 16, 17, 18, 31, and 32). Analysis of these data also suggested that the strain entered Japan independently of the aforementioned isolates. Strain NCTC9682, type 28, and the type of *S equi* isolated in Europe in the 1950s differed from isolates in Japan and major isolates in the United States but were closely related to isolates in Ireland, Scotland, Brazil, and Australia (types 25, 26, 27, and 29). This suggests that the *S equi* strains in Brazil and Australia may have originated in Europe.

a. STREP LA, Denka Seiken, Tokyo, Japan.
b. API 20 STREP, bioMerieuxsa, Marcy-l'Etoile, France.
c. Columbia agar base, BBL, Becton Dickinson Microbiology Systems, Sparks, Md.
d. Todd-Hewitt broth, Difco Laboratories Inc, Becton Dickinson Microbiology Systems, Sparks, Md.
e. InstaGene Matrix, Bio-Rad Laboratories, Hercules, Calif.
f. Applied Biosystems, Perkin-Elmer Inc, Foster City, Calif.
g. Info-SINCA, Fujitsu Kyushu System Engineering, Fukuoka, Japan.

References

1. Moore BO, Bryans JT. Antigenic classification of group C animal streptococci. *J Am Vet Med Assoc* 1969;155:416–421.
2. Galan JE, Timoney JF. Immunologic and genetic comparison of *Streptococcus equi* isolates from the United States and Europe. *J Clin Microbiol* 1988;26:1142–1146.
3. Jorm LR, Love DN, Bailey GD, et al. Genetic structure of populations of β-haemolytic Lancefield group C streptococci from horses and their association with disease. *Res Vet Sci* 1994;57:292–299.

4. Kuwamoto Y, Anzai T, Wada R. Microplate sugar-fermentation assay distinguishes *Streptococcus equi* from other streptococci of Lancefield's group C. *J Equine Sci* 2001;12:47–49.

5. Anzai T, Timoney JF, Kuwamoto Y, et al. In vivo pathogenicity and resistance to phagocytosis of *Streptococcus equi* strains with different levels of capsule expression. *Vet Microbiol* 1999;67:277–286.

6. Takai S, Anzai T, Yashiro H, et al. Detection of DNA restriction fragment polymorphism in *Streptococcus equi*. *Vet Rec* 2000;146:159–161.

7. Al-Ghamdi GM, Kapur V, Ames TR, et al. Use of repetitive sequence-based polymerase chain reaction for molecular epidemiologic analysis of *Streptococcus equi* subspecies *equi*. *Am J Vet Res* 2000;61:699–705.

8. Timoney JF, Artiushin SC, Boschwitz JS. Comparison of the sequences and functions of *Streptococcus equi* M-like proteins SeM and SzPSe. *Infect Immun* 1997;65:3600–3605.

9. Boschwitz JS, Timoney JF. Inhibition of C3 deposition on *Streptococcus equi* subsp. *equi* by M protein: a mechanism for survival in equine blood. *Infect Immun* 1994;62:3515–3520.

10. Meehan M, Nowlan P, Owen P. Affinity purification and characterization of a fibrinogen-binding protein complex which protects mice against lethal challenge with *Streptococcus equi* subsp. *equi*. *Microbiology* 1998;144:993–1003.

11. Meehan M, Muldowney DA, Watkins NJ, et al. Localization and characterization of the ligand-binding domain of the fibrinogen-binding protein (FgBP) of *Streptococcus equi* subsp. *equi*. *Microbiology* 2000;146:1187–1194.

12. Meehan M, Lynagh Y, Woods C, et al. The fibrinogen-binding protein (FgBP) of *Streptococcus equi* subsp. *equi* additionally binds IgG and contributes to virulence in a mouse model. *Microbiology* 2001;147:3311–3322.

13. Timoney JF, Umbach A, Boschwitz JE, et al. *Streptococcus equi* subsp. *equi* expresses 2 M-like proteins including a homologue of the variable M-like protective protein of subsp. *zooepidemicus*, in *Proceedings*. 7th Int Conf Equine Infect Dis. Newmarket, England: R & W Publications, 1994;189–193.

14. Anzai T, Nakanishi A, Wada R, et al. Isolation of *Streptococcus equi* subsp. *equi* from Thoroughbred horses in a racehorse-breeding area of Japan. *J Vet Med Sci* 1997;59:1031–1033.

15. Kimura M. The neutral theory of molecular evolution. In: Nei M, Koehn R, eds. *Evolution of genes and proteins*. Sunderland, Mass: Sinauer Associates. 1983;208–233.

16. Thompson J, Higgins DS, Gipson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680.

17. Saito N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.

18. Meehan M, Muldowney DA, O'Meara F, et al. Neither the A- nor B-repeat regions of the fibrinogen-binding protein of *Streptococcus equi* subsp. *equi* are essential for fibrinogen binding. *FEMS Microbiol Lett* 2000;190:317–321.

19. Chanter N, Talbot NC, Newton JR, et al. *Streptococcus equi* with truncated M proteins isolated from outwardly healthy horses. *Microbiology* 2000;146:1361–1369.

20. Timoney JF, Artiushin S, Wang J. Regions of the protective M-like protein of *Streptococcus equi* recognized by serum and mucosal antibodies of convalescent horses. In: Wernery U, Wade J, Mumford JA, et al, eds. *Equine infectious diseases VIII*. Newmarket, England: R & W Publications, 1999;88–94.

21. Katayama M, Miyama M, Furuya S, et al. Epidemiological analysis of a herd infected with strangles by imported carrier horse. *J Jpn Vet Med Assoc* 2003;56:139–143.

Appendix

Geographic location and year of isolation for *Streptococcus equi* used in the study.

Isolate	Geographic location	Year
Strain CF32	New York	1981
Strain Hidaka/95/2	Hidaka, Japan	1995
Strain NCTC9682	Europe	1950s
Isolate No. 1	Tokachi, Japan	1992
Isolate No. 2	Ishikari, Japan	1993
Isolate No. 4	Shiga, Japan	1994
Isolate No. 6	Kentucky	1990
Isolate No. 7	United States	Unknown
Isolate No. 8	Ireland	1983
Isolate No. 9	Ohio	1980
Isolate No. 10	New York	1980
Isolate No. 12	Unknown	1984
Isolate No. 13	Ireland	1983
Isolate No. 15	Hidaka, Japan	1995
Isolate No. 16	Hidaka, Japan	1996
Isolates Nos. 17–20	Tokachi, Japan	1996
Isolates Nos. 21–23	New York	1983
Isolates Nos. 24–27	Kentucky	1984
Isolates Nos. 28–30	Kentucky	1998
Isolates Nos. 31 and 32	New Jersey	1998
Isolate No. 33	Ireland	1983
Isolate No. 34	Kentucky	1990
Isolate No. 35	New York	1989
Isolate No. 36	New York	1981
Isolate No. 37	New York	1976
Isolate No. 38	New York	1980
Isolate No. 39	Ireland	1983
Isolate No. 40	Minnesota	1995
Isolate No. 41	Kentucky	1993
Isolates Nos. 42–44	Scotland	1991
Isolate No. 45	New York	1975
Isolate No. 46	Minnesota	1996
Isolates Nos. 47 and 48	Minnesota	1996
Isolates Nos. 49 and 50	Minnesota	1996
Isolate No. 51	Minnesota	1996
Isolate No. 52	Kentucky	1997
Isolates Nos. 53 and 54	Kentucky	1997
Isolate No. 55	Australia	1990
Isolate No. 56	Kentucky	1995
Isolate No. 57	Kentucky	1993
Isolate No. 58	New York	1976
Isolate No. 59	New York	Unknown
Isolate No. 60	Kentucky	1987
Isolates Nos. 61 and 62	Kentucky	1988
Isolate No. 64	Sweden	1982
Isolate No. 65	Kentucky	1994
Isolate No. 66	New York	1987
Isolate No. 67	Kentucky	1997
Isolate No. 68	New York	Unknown
Isolate No. 69	New York	1980
Isolate No. 70	Kentucky	1997
Isolates Nos. 76 and 77	Tokachi, Japan	1998
Isolates Nos. 78 and 79	Brazil	1999
Isolates Nos. 80–83	Hidaka, Japan	1999
Isolates Nos. 84–86	Hidaka, Japan	1999
Isolates Nos. 87 and 88	Hidaka, Japan	2000
Isolates Nos. 89–91	Hidaka, Japan	2000
Isolate No. 92	Chiba, Japan	2001