Polymerase chain reaction-restriction fragment length polymorphism analysis of the *SzP* gene of *Streptococcus zooepidemicus* isolated from the respiratory tract of horses

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Objective—To develop polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis for molecular typing of strains of *Streptococcus zooepidemicus* and to use the new typing method to analyze a collection of isolates from the respiratory tract of Thoroughbreds.

Sample Population—10 strains of *S zooepidemicus*, 65 isolates from the respiratory tract of 9 yearlings following long distance transportation, and 89 isolates from tracheal aspirates of 20 foals with pneumonia.

Procedure—Phenotypic variations in the SzP protein were detected by western immunoblot analysis. Using PCR-RFLP analysis, genotypes were obtained with primer sets from the *SzP* gene, followed by restriction endonuclease digestion of the amplicons.

Results—Unique genotypic patterns were obtained with a primer set designed from both ends of the structural gene and the restriction endonuclease *Dde* I. Forty-five isolates from the lymphoid tissue within the pharyngeal recess (ie, pharyngeal tonsil) of yearlings included 10 *SzP* genotypes and SzP phenotypes. Isolates from the trachea of each yearling were of a single genotype that was also present among isolates from the pharyngeal tonsil of the same horses. Isolates from tracheal aspirates of foals belonged to 14 genotypes.

Conclusions and Clinical Relevance—Analysis of the *SzP* gene by use of PCR-RFLP was effective for molecular typing of strains of *S zooepidemicus* in the study of respiratory tract disease in horses. Results of PCR-RFLP analysis indicate that a single strain of *S zooepidemicus* can migrate from the pharyngeal tonsil to the trachea at a high rate in horses undergoing long distance transportation. (*Am J Vet Res* 2002;63:1298–1301)

Streptococcus zooepidemicus (*S equi* subsp *zooepidemicus*) is the most common opportunist infectious organism of the respiratory tract in horses. A usual commensal of the lymphoid tissue within the pharyngeal recess (ie, pharyngeal tonsil) and nasopharyngeal mucosa, *S zooepidemicus* commonly invades the lower portion of the respiratory tract of horses that is stressed by prolonged transportation and causes severe, sometimes fatal, pleuropneumonia.[1,2] Purulent rhinitis and bronchitis in weanling foals are also caused by *S zooepidemicus* infection, resulting in serious losses because of impaired growth and the cost of veterinary care.[3,4]

In a previous study,[5] we compared *S zooepidemicus* isolates from pharyngeal tonsils of healthy horses with isolates from foals and donkeys with pneumonia with respect to phenotype and genotype of the SzP protein, a major protective and antigenic M-like protein of *S zooepidemicus*. On the basis of the type of SzP protein, the same *S zooepidemicus* isolates found in pharyngeal tonsils of healthy horses were represented among isolates from foals and donkeys with pneumonia. These findings indicate that streptococcal pneumonia in equids is an infection that involves invasion by a single endogenous clone or strain of *S zooepidemicus*.

The SzP protein is a mouse protective and antiphagocytic M-like protein that is expressed on the cell surface of *S zooepidemicus*. The SzP protein is the typing antigen of Moore and Bryans[6] serovars. Variability of the SzP protein is mainly dependent on a varied amino acid sequence in 3 separate regions: the N-terminus, a central hypervariable region, and the PEPK repeat region in the carboxyterminus.[7] Analysis of the SzP protein by use of western immunoblot technique or by DNA sequencing has been used for an epidemiologic survey and for investigation of the mechanism of infection.[5,8] However, this complex approach is not convenient or practical. The purpose of the study presented here was to develop a simpler method, **polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)** analysis, for the molecular typing of strains of *S zooepidemicus* and then to use the new typing method to analyze a collection of isolates from the respiratory tract of Thoroughbreds transported long distance or with pneumonia.

Materials and Methods

Bacteria—In our study, 10 strains of *S zooepidemicus*, for which the amino acid sequence of the SzP proteins had previously been determined, were used as standards. *Streptococcus zooepidemicus* W60, isolated from a nasal discharge of a horse in the United States in 1976, is the prototype strain of *S zooepidemicus*, from which the *SzP* gene was first sequenced.[9] Strains 1, 3, 7, 521, 2123, 3936, 6708, 6729,

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and 29394 of *S zooepidemicus* were isolated from donkeys with pneumonia secondary to influenza infection and from foals with pneumonia in the United States in 1994.[5] Amino acid sequences of proteins of these strains of *S zooepidemicus* have been described (**Fig 1**).[5]

Sixty-five isolates of *S zooepidemicus* were recovered from the respiratory tract of 9 yearling Thoroughbreds following long distance transportation in Japan. These isolates were obtained from 9 pharyngeal tonsil and 4 tracheal mucosal swab specimens following euthanasia for reasons unrelated to respiratory tract disease. Swab specimens were plated on Colombia CNA agar[a] supplemented with 5% heparinized horse blood for bacteriologic culture. Five *S zooepidemicus* colonies were picked from bacteriologic cultures and identified by use of a slide latex agglutination kit for streptococcal grouping[b] and a commercial identification system for *Streptococcus*.[c]

From 20 foals in Japan with pneumonia, 89 isolates of *S zooepidemicus* were obtained from tracheal aspirates. Two to 5 colonies of *S zooepidemicus* were isolated from each specimen and identified.

All 154 isolates were stored at −80°C. A colony of each isolates on Colombia agar with 5% horse blood was subcultured in 1 mL of Todd Hewitt broth[a] at 37°C for 18 hours, centrifuged at 15,000 × *g* for 1 minute, and the supernatant removed. Cell pellets were used as a source of bacterial protein and DNA.

Immunoblot analysis of the SzP protein—Cell surface proteins were extracted with mutanolysin and lysozyme from the cell pellet as described.[9] Proteins in the extract were separated by SDS-PAGE on 12% polyacrylamide and transferred electrophoretically to nitrocellulose membranes. Immunoblots were developed in rabbit antiserum specific for recombinant SzPW60 from *S zooepidemicus* W60.[7]

PCR-RFLP analysis of the *SzP* gene—Two hundred microliters of a commercial matrix for preparation of PCR template DNA[d] was added to the bacterial cell pellet, and genomic DNA was extracted according to the manufacturer's instructions. The PCR analysis was performed in 100 µL-volumes containing 1 µL of genomic DNA, 20m*M* Tris-HCI (pH 8.0), 100m*M* KCI, 3m*M* $MgCl_2$, 200 µ*M* (each) deoxynucleoside triphosphates, 0.2 µ*M* (each) of primer, and 2.5 U of a PCR enzyme[e]. N (5'ACAAAAGGGGAATAAAATGGC) and NCR4 (5'TTTACCACTGGGGTATAAGGCTT) primers[7] were used to amplify the *SzP* gene. Reactions were performed in a PCR thermal cycler[f] programmed as follows: 3 minutes at 98°C, 30 cycles of 5 s/cycle at 55°C, 10 seconds at 72°C, 1 second at 98°C, and 3 minutes at 72°C. The amplicon was purified by use of a spin column chromatography system,[g] and 50 µL of the purified solution, 50 µL of H_2O, 3.3 µL of 3*M* sodium acetate, and 250 µL of ethanol were mixed by a vortex mixer. Following removal of the supernatant after centrifugation at 18,000 × *g* for 5 minutes, the precipitate was dried by vacuum centrifugation following a single rinse in ethanol. Eight kinds of restriction endonucleases[h] suitable for RFPL analysis of the *SzP* gene were chosen by use of the SzPW60 DNA sequence. Each restriction endonuclease in a 40-µL solution was added to a tube of dried precipitate and the DNA digested as described. Analysis of DNA fragments was performed by 3.5% agarose gel electrophoresis.

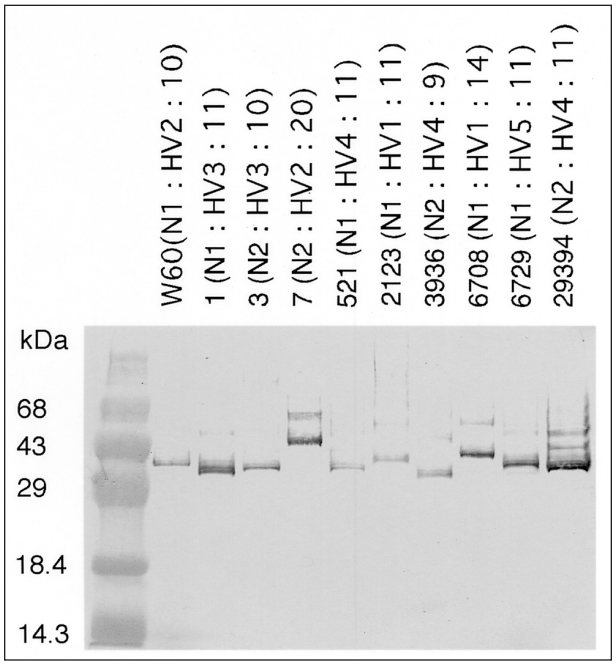

Figure 1—Immunoblot reactions of mutanolysin extracted proteins with SzPW60 specific antiserum for 10 isolates of *Streptococcus zooepidemicus* after separation by SDS-PAGE. Molecular masses of protein standards are indicated in kilodaltons at the left side of the figure. Designation and amino acid sequence motifs of each isolate are above the figure.

Results

Using PCR analysis with the N-NCR4 primer set, DNA fragments of approximately 1,100 **base pairs (bp)** were amplified from all strains of *S zooepidemicus* originating from the United States (standards) and Japan. Because all of the restriction endonucleases were predicted to cleave the *SzPW60* gene in at least 5

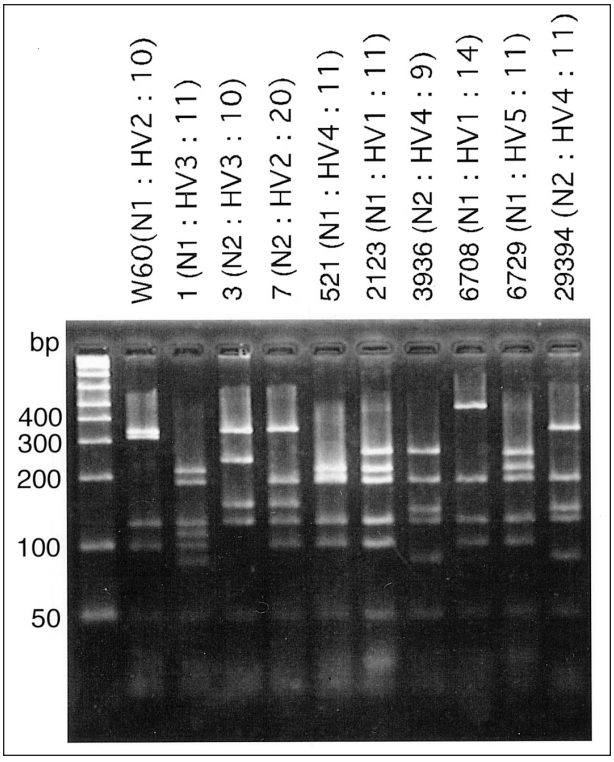

Figure 2—Deoxyribonucleic acid fragments produced following polymerase chain reaction-restriction fragment length polymorphism analysis of the *SzP* gene in 10 isolates of *Streptococcus zooepidemicus* that originated in the United States. Deoxyribonucleic acid size markers in base pairs are indicated on the left side of the figure. Designation and amino acid sequence motifs of each strain of *S zooepidemicus* are above the figure.

sites, the restriction fragment profiles for each restriction endonuclease were compared for 10 strains of *S zooepidemicus* originating from the United States. Four or 5 fragments of 60 to 400 bp that varied in size for each strain of *S zooepidemicus* were generated by use of *Dde*I (**Fig 2**). Because a smaller number of fragments were produced by use of the other restriction endonuclease, *Dde*I was chosen to screen isolates from horses in Japan.

Ten phenotypes and genotypes were distinguished among 45 strains of *S zooepidemicus* isolated from pharyngeal tonsils of 9 yearling Thoroughbreds by use of immunoblotting or by use of PCR-RFLP analysis. Both molecular typing methods produced similar results (**Table 1**). Twenty isolates from the tracheal mucosa were of 4 types, each of which was identical to an isolate in the pharyngeal tonsil of the same horse. A total of 89 isolates from 20 Thoroughbred foals were sorted into 14 genotypes by use of PCR-RFLP analysis, 6 of which were identical to genotypes in the pharyngeal tonsil of the yearling Thoroughbreds (**Table 2**).

Table 1—SzP phenotype and genotype of 65 *Streptococcus zooepidemicus* isolates* from tonsils and tracheal swab specimens from 9 yearling Thoroughbreds following transportation

Horse	No. of isolates: Pharyngeal tonsil	No. of isolates: Trachea	SzP phenotype	*SzP* genotype (fragment sizes**)
1	5	ND	I	*I* (210, 190, 130, 120, 100, 90)
	3	5	II	*II* (290, 190, 180, 130, 90)
2	1	ND	I	*I*
	1	ND	III	*III* (250, 210, 190, 130, 100)
	2	ND	IV	*IV* (390, 210, 130, 100)
3	2	ND	V	*V* (250, 190, 150, 135, 130)
	1	5	VI	*VI* (295, 290, 130, 100)
4	5	5	I	*I*
	3	ND	VII	*VII* (350, 290, 130, 120)
5	2	ND	VIII	*VIII* (360, 350, 130, 100
6	5	ND	I	*I*
7	5	5	I	*I*
8	4	ND	IV	*IV*
	1	ND	IX	*IX* (350, 290, 130, 100, 65)
9	5	ND	X	*X* (295, 290, 130, 100, 65)

*Five isolates were obtained from each specimen.
**Fragment sizes between 390–65 base pairs were estimated in comparison with a DNA size marker.
ND = β-haemolytic colonies were not detected on CNA blood agar.

Table 2—*SzP* genotypes of 89 isolates* of *Streptococcus zooepidemicus* from tracheal aspirates of 20 Thoroughbred foals with pneumonia

SzP genotype (Fragment sizes**)	No. of isolates
I	9
II	2
IV	32
VI	5
VIII	2
IX	5
XI (350, 190, 130, 120, 90)	10
XII (290, 190, 180, 130, 100)	6
XIII (190, 135, 125, 90, 85,)	5
XIV (210, 195, 190, 130, 100)	2
XV (350, 190, 135, 130, 90)	1
XVI (290, 190, 150, 130)	4
XVII (190, 135, 130, 90, 85)	1
XVIII (350, 290, 130, 100)	5

*Approximately 5 isolates were examined from each specimen.
** Fragment sizes between 390–65 bp were estimated in comparison with a DNA size marker.

Discussion

Moore and Bryans[6] serovars of *S zooepidemicus* were established by precipitin analysis of acid extracts of a series of 164 isolates from horses in Kentucky. Of these isolates, 68 were typeable as 1 of a set of 13 serotypes.[6] Results of a subsequent study[9] revealed that the typing antigen in *S zooepidemicus* W60 was an acid resistant fibrillar protein of molecular weight, 40,123. This protein was designated SzPW60[9] and was mouse protective, opsonogenic, and M-like. Considerable variation was found in the amino acid sequence of the SzP proteins in a series of isolates originating from the United States, including the Moore and Bryans[6] serovars.[7,10] Variant motifs have been of value in understanding the pathogenesis of pneumonia in horses[5] and in determining the source of infection and immune responses of humans in an outbreak of poststreptococcal infection glomerulonephritis.[8] Analysis by PCR-RFLP clearly simplifies the use of the variant SzP proteins in epidemiologic and pathogenic studies of *S zooepidemicus* infection because PCR-RFLP analysis is rapid, involves fewer steps than a combination of PCR analysis and sequencing, and is not dependent on availability of hyperimmune serum. Using PCR-RFLP analysis, a similarity between SzP proteins of *S zooepidemicus* isolates originating from Japan and the United States was detected, indicating universal applicability of the technique.

Although 17 *Dde*I cleavage sites (CTNAG) were found in the *SzPW60* gene, all but 2 of these were concentrated in the 5' region of the gene. Most sites were in the PEPK repeat region of the predicted protein, resulting in production of fragments that were too small to be detected by agarose gel electrophoresis. The largest (335 bp) fragment included much of the hypervariable central region.[7] The next largest comprised the N-terminal variable region and portion of the hypervariable central region. Thus, variability in size was greater for amplicons in the size range 100 to 400 bp than for smaller fragments (Fig 2).

It has been suggested that pneumonia in horses, which is caused by a *S zooepidemicus* infection, originate from endogenous clones in the pharyngeal tonsil. This hypothesis stems from the observation that the phenotype of the SzP protein of *S zooepidemicus* isolated from pharyngeal tonsils of healthy horses is the same as in organisms isolated from pneumonic lungs. Results of this study confirm the observation. The *SzP* genotypes of *S zooepidemicus* isolates from the pharyngeal tonsils of horses were also represented among isolates from tracheal aspirates of foals with pneumonia. Furthermore, although a variety of *SzP* genotypes were found in isolates from the pharyngeal tonsils of 9 yearling Thoroughbreds, only 1 genotype was found in the trachea of each of 4 horses following transportation. For each horse, the genotype of the clone found in the trachea was also represented in the pha-

ryngeal tonsil. Colonization of the trachea may represent the first stage in invasion of the lower portion of the respiratory tract, because these 4 horses did not have pneumonia.

[a]Becton Dickinson Microbiology Systems, Md.
[b]STREPT LA, bioMerieuxsa, Marcy-l'Etoile, France.
[c]API 20 STREP, Denka Seiken, Tokyo, Japan.
[d]InstaGene Matrix, Bio-Rad Laboratories, Calif.
[e]Z-Taq, Takara Shuzo Biomedical Group, Shiga, Japan.
[f]TaKaRa PCR Thermal Cycler MD, Takara Shuzo Biomedical Group, Shiga, Japan.
[g]MicroSpin S-400 HR, Amersham Pharmacia Biotech, Buckinghamshire, England.
[h]*Alu*I, *Bbv*I, *Bfa*I, *Dde*I, *Mbo*II, *Mwo*I, *Sau*3AI, and *Tsp*509I, New England BioLabs, Mass.

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