Comparison of the phenotypes of *Streptococcus zooepidemicus* isolated from tonsils of healthy horses and specimens obtained from foals and donkeys with pneumonia

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Objective—To determine whether streptococcal pneumonia is caused by strains of *Streptococcus zooepidemicus* similar to those obtained from the tonsils of healthy horses.

Sample Population—5 tonsils from healthy horses, 8 tracheal washes and 6 lung specimens from foals with pneumonia, and 5 nasopharyngeal swab specimens from donkeys with acute bronchopneumonia.

Procedure—Variable M-like protectively immunogenic SzP proteins of 5 isolates of *S zooepidemicus* from each tonsil and clinical specimen were compared, using immunoblots. The *SzP* gene of 13 isolates representative of various SzP immunoblot phenotypes from 1 healthy horse and 9 horses and donkeys with pneumonia were sequenced and compared. Cell-associated hyaluronic acid concentration and resistance to phagocytosis of some isolates were measured.

Results—Tonsils of each healthy horse were colonized by several SzP phenotypes similar to those of foals or donkeys with pneumonia. In contrast, multiple isolates from animals with pneumonia had the same SzP phenotype, indicating infection by a single strain or clone. Analysis of the SzP sequence confirmed that differences in immunoblot phenotype were associated with sequence differences and that several SzP genotypes were in healthy horses and animals with pneumonia. Isolates with high concentrations of cell-associated hyaluronic acid were more resistant to phagocytosis.

Conclusions and Clinical Relevance—An SzP-specific immunoblot is a useful, sensitive measure of diversity among strains of *S zooepidemicus*. Single strains with SzP phenotypes similar to those found in tonsils of healthy horses cause pneumonia. Because of the diversity of SzP phenotype and genotype among isolates from animals with pneumonia, SzP phenotype is not an important determinant of invasiveness or epizootic capabilities. (*Am J Vet Res* 2000;61:162–166)

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Streptococcus zooepidemicus (Lancefield group C) is a commensal organism of the equine tonsil and nasopharyngeal mucosa that may opportunistically invade the respiratory tract, causing purulent rhinitis and bronchitis in weanling foals and pneumonia in horses and donkeys of all ages.[1-5] It is also an important cause of acute hemorrhagic pneumonia in older horses that have been stressed by prolonged transportation.[6] Equine strains of *S zooepidemicus* have great antigenic variation of an acid- and heat-resistant, trypsin-sensitive, α-helical, M-like protein (ie, SzP).[7,8] This protein is the Moore and Bryans typing antigen, is opsonogenic and mouse-protective, and can vary at its amino N-terminus and in a central **hypervariable (HV)** region.[9,10] Additional variability is found in a proline-rich region close to the membrane anchor wherein there may be a variable number of **proline-glutamic acid-proline-lysine (PEPK)** repeats. The SzP proteins of the Moore and Bryans antigen-typed serovars have differing characteristic immunoblot band patterns after **sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** and reaction with SzP-specific antiserum.[9,10] This variation, together with the aforementioned sequence differences, are potentially useful in recognition of strains of *S zooepidemicus* in clinical specimens and tissues of healthy horses.

In a study[7] based on the use of acid-extracted antigens of *S zooepidemicus*, serotype was unrelated to clinical source of specimens obtained from the lungs, joints, uterus, fetus, lymph nodes, semen, or other sites in horses. Moreover, samples from diseased respiratory tracts collectively contained several serotypes. These findings pointed to the opportunistic nature of *S zooepidemicus* infections in horses and provided little support for the existence of specific strains during outbreaks of respiratory tract disease. Almost all horses harbor a number of antigenic types of *S zooepidemicus* in their tonsils,[11] and it has been suggested that this site is the source of opportunistic infections of the lung and airways, as seen in equids stressed by high temperature (ie, summer pneumonia), transportation, or a recent influenza infection.[2,6,12] In the study reported here, immunoblot analysis and evaluation of the amino acid sequence of the highly variable SzP protein were used to test the hypothesis that invasions of the lung and airways in horses and donkeys are caused by single strains of *S zooepidemicus* and not by a mixture of strains as may be predicted from detection of multiple antigenic types in the tonsils of healthy horses.[11]

Materials and Methods

Bacteria—Strains of *S zooepidemicus* were obtained from various sources. Horses that were sources of tonsillar isolates and donkeys experimentally infected with equine influenza virus were housed in separate fields at a university equine farm. Specimens from foals with pneumonia were obtained in the summer of 1994 from an animal disease diagnostic laboratory[a] or from equine veterinary practices located near Lexington, Ky.

Lingual tonsillar tissues were collected during necropsy from 5 healthy yearling horses. Each tonsil was incised through its center, and a specimen from the freshly cut surface was cultured on **colistin-nalidixic acid (CNA)** horse blood agar. Nasopharyngeal swab specimens obtained from 5 adult donkeys with pneumonia secondary to influenza also were cultured on CNA agar. All 5 donkeys had severe bronchopneumonia secondary to experimentally induced intranasal infection 7 days after inoculation with 1.3×10^6 plaque-forming units of influenza A/equine-2 (KY/1/91, H3 N8) virus.[b] Lung tissue from 6 foals with pneumonia that died as a result of natural infection with *S zooepidemicus* and tracheal washes obtained from 8 other foals with summer pneumonia also were cultured on CNA agar. After incubation at 37 C for 18 hours, 5 β-hemolytic colonies from each plate were cultured separately on CNA agar. Identification of each colony was made on the basis of fermentation reactions in lactose, sorbitol, and trehalose and was confirmed by inoculation of commercially available identification strips.[c] Cultures for extraction of surface proteins were incubated in 10 ml of **Todd Hewitt broth (THB)**[d] at 37 for 18 hours. Cultures then were centrifuged, and the cell pellet was extracted with mutanolysin and lysozyme, as described elsewhere.[8]

Immunoblot analysis—Proteins in extracts of each clone were separated by SDS-PAGE, using a 12% polyacrylamide gel. Extracted proteins were transferred electrophoretically to nitrocellulose.[10] Immunoblots were developed in rabbit 191 antiserum to prototype recombinant SzPW60 from *S zooepidemicus* W60.[8]

Sequence analysis of M-like protein genes—Nucleotide sequences of *SzP* genes of 4 tonsillar specimens from healthy horses, 3 specimens from donkeys with experimentally induced pneumonia, and 6 isolates of *S zooepidemicus* cultured from specimens obtained from foals with pneumonia were considered representative of each of the immunoblot patterns; nucleotide sequences were determined in accordance with the procedure of Walker and Timoney.[10] The M-like genes were amplified from genomic DNA extracts in a **polymerase chain reaction (PCR)** by use of coding primer 3 (5'-ACAAAAGGGGAATAAAATGGC-3') and noncoding primer 3 (5'-TTTACCACTGGGGTATAAGGC-3') determined from the sequence of the prototype *SzP* gene.[9,10] The PCR consisted of 2.5 µl of 10× buffer[d] (2.5 µl each of 5 µ*M* forward and reverse primers), 2 µl of 10 m*M* dNTP,[e] 1.5 µl of $MgCl_2$, 0.3 µl of *Taq* polymerase,[e] 1 µg of chromosomal DNA, and a sufficient amount of distilled water to achieve a final volume of 25 µl. Reactions were performed in a thermocycler[f] programmed as follows: 2 minutes at 95 C, 25 cycles of 1 min/cycle at 50 C, 2.5 minutes at 72 C, 1 minute at 95 C, and 5 minutes at 72 C. The PCR products of approximately 1.1 kilobases were purified, using a commercial PCR preparation kit,[g] ligated into a commercial plasmid vector,[h] and transformed into *Escherichia coli*.[h] Potential positive colonies (white) on L-agar supplemented with ampicillin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (50 µg/ml) were confirmed by PCR analysis, using coding and noncoding primers 3 as described earlier. Plasmid DNA was then extracted, using a commercial resin chromatograph kit,[g] and insert DNA was sequenced[i] initially with universal sequencing primers and subsequently (as necessary) with internal coding primers P11 (5'-TTTGGGCTAGCTTCAGTATCGG-3'), P8 (5'-ATTTGACACCGCTCAAGCAGACC-3'), and P4 (5'-AGAGATGATCTTAGACTCAATTGC-3') and noncoding primers P10r (5'-TCTAAGATCATCTCTGAGTG-3') and P13r (5'-TCTTCGATACCAGCATAAGCTC-3') from the prototype SzP W60 gene, as described elsewhere.[9] Nucleotide sequence was translated into amino acid sequence by use of a computer sequencing program.[13]

Measurement of cell-associated hyaluronic acid—Concentration of cell-associated hyaluronic acid during the logarithmic growth phase in cultures of isolates from 3 donkeys, 2 foals with pneumonia that died, and prototype *S zooepidemicus* W60 was measured. After overnight culture in THB at 37 C, aliquots (200 µl) were added to 10 ml of THB supplemented with 10% horse serum and incubated for 3 hours at 37 C. Bacteria obtained during the logarithmic growth phase were pelleted by centrifugation at 8,000 × g for 10 minutes and then washed with phosphate-buffered saline solution, pH 7.4. cells were suspended in 2.0 ml of a solution containing equal volumes of water and chloroform, mixed vigorously, and allowed to stand at room temperature for 1 hour. After centrifugation, the carbazole assay[14] was performed on the aqueous phase, using hyaluronic acid from *S zooepidemicus*[j] as the basis for a standard curve. The control sample consisted of ethanol in place of carbazole reagent.

Assay of opsonophagocytic activity—Neutrophils were collected from 6 ml of heparinized blood obtained from a healthy pony, using a discontinuous Percoll gradient,[15] and suspended in 2 ml of Hank's balanced salt solution.[i] Isolates obtained during the logarithmic growth phase from the aforementioned specimens were prepared as described and suspended at a final concentration of approximately 1×10^7 colony-forming units/ml of Hank's balanced salt solution; 25 µl of each suspension was opsonized by use of 25 µl of rabbit 191 antiserum to recombinant SzP W60 for 5 minutes. Aliquots (200 µl) of neutrophil suspension (10^6 cells) were mixed with the opsonized bacterial suspension to provide a neutrophil-to-bacteria ratio of approximately 3:1, and these mixtures were rotated at 37 C for 90 minutes. Two aliquots (10 µl) of the neutrophil-bacteria suspension were removed, mixed with 13-ml aliquots of molten tryptic soy agar at 45 C containing 5% heparinized horse blood, and poured onto Petri dishes. After overnight incubation at 37 C, colony counts were performed. A Student *t*-test (one-tailed) was used to determine whether colony counts at 90 minutes for isolates with high concentrations of cell-associated hyaluronic acid were significantly higher than colony counts for isolates with low concentrations. Values of $P < 0.05$ were considered significant.

Results

Bacterial culture—Isolates from all specimens cultured on CNA blood agar were dominated by β-hemolytic colonies. In the case of specimens from 2 donkeys, only mucoid colonies were observed, suggesting infection by an encapsulated strain. Mucoid and nonmucoid colonies were observed in cultures of specimens from 3 other donkeys. The β-hemolytic colonies in cultures of tonsillar specimens were nonmucoid and varied in shape, texture, and size. In contrast, colonies in a culture of a specimen from each foal with pneumonia were similar. Information concerning the mucoid character of these colonies immediately after primary culture was not available. Five β-hemolytic colonies, which were randomly selected from each primary culture and subcloned on CNA agar, fermented lactose and sorbitol but not tre-

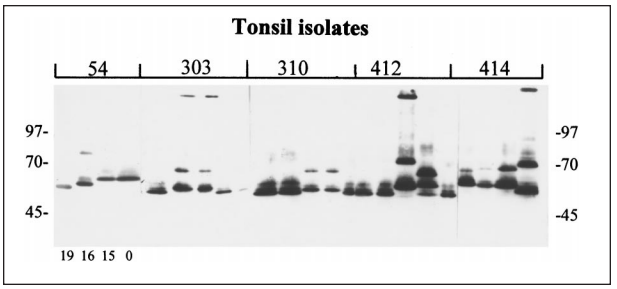

Figure 1—Composite immunoblot of the reaction of mutanolysin-extracted proteins in *Streptococcus zooepidemicus* isolated from tonsils of 5 healthy horses after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transfer to nitrocellulose, and development in rabbit 191 antiserum to recombinant SzPW60.[8] Molecular masses of protein standards are indicated in kilodaltons at each side of the figure. Numbers below the figure are the isolates from the tonsils of a healthy horse for which the SzP sequences were reported in Table 1.

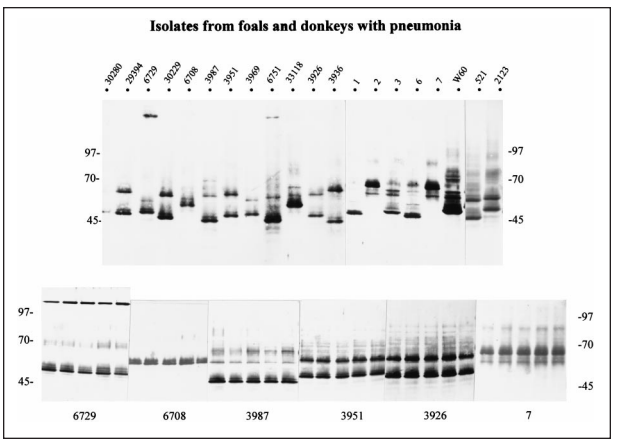

Figure 2—Composite immunoblot of the reactions of SzP proteins in mutanolysin extracts *of S zooepidemicus* from foals (30280, 29394, 6729, 30229, 6708, 3987, 3951, 3969, 6751, 33118, 3926, 3936, 521, 2123) and donkeys (1, 2, 3, 4, 6, 7) with pneumonia. The lane identified as W60 indicates the reaction of SzPW60 for the prototype strain *S zooepidemicus* W60. The lower panel depicts the reactions of sets of 5 isolates from each of the 6 representative animals indicated. Molecular masses of protein standards are indicated in kilodaltons at each side of the figure.

halose and, therefore, presumptively were identified as *S zooepidemicus*. This was confirmed by analysis of results for a commercial streptococcal-specific identification system.[c]

Immunoblot analysis—Immunoblot reactions were determined for the SzP proteins of *S zooepidemicus* isolates cultured from the tonsils of 5 healthy yearling horses (**Fig 1**). On the basis of the pattern and size of reactive bands, at least 2 SzP phenotypes were detected among the 5 *S zooepidemicus* isolates cultured from a single tonsil. Seven phenotypes of SzP were detected among the isolates cultured from the tonsils of the 5 horses. Immunoblot patterns were determined for isolates from nasal swab specimens of donkeys with bronchopneumonia secondary to experimentally induced influenza virus infection (**Fig 2**). An identical SzP phenotype was evident for all isolates tested from 2 donkeys, whereas 2 other donkeys were infected with a strain that had another phenotype, and still another donkey had a strain with a third SzP phenotype. All isolates cultured from a particular donkey had identical SzP phenotypes.

The SzP proteins were determined for *S zooepidemicus* isolates cultured from specimens obtained from each foal with pneumonia (Fig 2), which illustrated the diversity of SzP phenotypes in this group of isolates. However, isolates from each foal were identical, except for 1 foal in which 4 of 5 isolates were identical (data not shown). Molecular weight of the most reactive band in each of the extracts varied from about 56,000 to 70,000. In 3 extracts, 2 strongly reactive bands were evident. Isolates from 4 foals appeared to be identical; similarly, isolates from the other 2 foals appeared to be identical.

Amino acid sequences—The PCR performed by use of coding and noncoding primers 3 was effective in amplifying DNA corresponding to the *SzP* genes of all 13 isolates cultured from tonsils of healthy horses and donkeys and foals with pneumonia that were selected for amino acid sequence analysis (**Table 1**). The amino acid sequence predicted for the N-terminal and central HV regions and number of PEPK repeats for the SzP proteins of the 13 isolates were determined. There were 3

Table 1—Variability in the SzP proteins of representative *Streptococcus zooepidemicus* isolates from the tonsils of a healthy horse and from specimens obtained from foals and donkeys with pneumonia. Amino acid sequences of the N terminus, hypervariable (HV) central region, and proline-glutamic acid-proline-lysine (PEPK) repeat regions were predicted from DNA obtained by use of polymerase chain reaction and primers from the prototype gene SzPW60[8,10]

Isolate	Origin	N Terminus	HV Motif	% Homology*	No. of PEPK repeats
54–0	Tonsil—healthy horse	N2	HV2	100 (MB1)	11
54–15	Tonsil—healthy horse	N2	HV2	100 (MB1)	11
54–16	Tonsil—healthy horse	N1	HV3	95 (MB5)	11
54–19	Tonsil—healthy horse	N2	HV4	100 (MB8)	11
1	Donkey with pneumonia	N1	HV3	95 (MB5)	11
3	Donkey with pneumonia	N2	HV3	79 (MB5)	10
7	Donkey with pneumonia	N2	HV2	94 (MB1)	20
521	Foal with pneumonia	N1	HV4	82 (MB8)	11
2123	Foal with pneumonia	N1	HV1	100 (MB9)	11
3936	Foal with pneumonia	N2	HV4	97 (MB8)	9
6708	Foal with pneumonia	N1	HV1	88 (MB9)	14
6729	Foal with pneumonia	N1	HV5	96 (MB10)	11
29394	Foal with pneumonia	N2	HV4	92 (MB8)	11

*Compared with the HV sequence of the SzP protein of the Moore and Bryans (MB) serovar[10] (indicated in parenthesis).

Table 2—Concentration of cell-associated hyaluronic acid (HA) and resistance to phagocytosis by equine neutrophils for 90 minutes for *S zooepidemicus* isolates from foals and donkeys with pneumonia

Identification	Source	Mucoid colony	μg of HA/g of cells	No. of colonies*	
				0 min	90 min
1	Donkey with pneumonia	No	706	526, 680	553, 578[a]
3	Donkey with pneumonia	Yes	2,256	186, 287	136, 284[a]
7	Donkey with pneumonia	Yes	1,139	247, 291	411, 296[a]
521	Foal with pneumonia	No	177	396, 429	3, 32[b]
2123	Foal with pneumonia	No	131	141, 274	1, 10[b]
W60	Prototype strain	No	136	231, 247	2, 1[b]

*Values reported are means of triplicate counts from 2 experiments.
[a,b]After 90 min of phagocytosis, No. of colonies for isolates with low concentrations of cell-associated HA was significantly ($P = 0.034$) lower than No. of colonies for isolates with high concentrations of cell-associated HA.

SzP sequences found for isolates from tonsillar specimens from one of the horses, which was consistent with the 3 phenotypes evident on immunoblot analysis of SzP proteins. The SzP proteins of 3 of these isolates had N2 N-terminal sequences, whereas another isolate had an N1 sequence. The HV regions of these SzP proteins were HV2 (2 isolates), HV3 (1), and HV4 (1). Thus, considerable variability in the SzP sequence was evident in *S zooepidemicus* flora obtained from the tonsil of this one horse. Considered as a group, the SzP proteins of isolates from donkeys and foals with pneumonia also had great sequence variation. The N1 and N2 termini were about equally represented and were combined in each isolate with the 5 known HV sequences (HV1 to HV5). The HV sequences of the SzP proteins of 2 isolates (1 donkey and 1 foal with pneumonia) were similar to those of 2 of the aforementioned tonsillar isolates from the healthy horse. Number of PEPK repeats identified in isolates from animals with pneumonia varied from 9 to 20. However, there were 11 repeats in 5 of the 9 SzP proteins sequenced. The assignment of HV motif was made on the basis of percentage of homology of the sequence (residues 117 to 163) with the appropriate HV motif of the Moore and Bryans serovars.[10] In all comparisons, homology was better than 79%. Number of amino acids that differed in each comparison ranged from 0 (a foal with pneumonia) to 8 (a donkey with experimentally induced pneumonia).

Cell-associated hyaluronic acid and opsonophagocytic assays—Amount of cell-associated hyaluronic acid varied from 2,256 μg/g in cells for an isolate from 1 donkey to 131 μg/g in cells for an isolate from 1 foal. As expected, isolates producing mucoid colonies had high concentrations of cell-associated hyaluronic acid and were resistant to phagocytosis (**Table 2**).

Discussion

The number of SzP immunoblot phenotypes and SzP sequences observed among isolates from tonsils of healthy horses is in marked contrast to the uniformity of multiple isolates from a single foal or donkey with pneumonia. This observation in combination with the fact that all the SzP phenotypes found in tonsils of healthy horses were represented among the isolates from foals and donkeys with pneumonia strongly favors the hypothesis that streptococcal pneumonia in equids is an endogenous infection that involves invasion by a single clone or strain of *S zooepidemicus*. It is unclear whether invasion of the bronchi and lungs results from failure of the mucociliary escalator to remove *S zooepidemicus* aspirated from the pharynx or whether the number of organisms of a single *S zooepidemicus* strain becomes so excessive in the nasopharynx that it overloads the normally efficient clearance mechanisms of the bronchi and bronchioles after aspiration of a bacteria-laden airstream. Kamada and Akiyama[16] reported that the distribution and behavior of *S zooepidemicus* in the bronchi are markedly influenced by the number of bacteria in the tonsils. Oikawa et al[6] subsequently observed that acute lacunar tonsillitis is a feature of hemorrhagic pneumonia in recently transported horses and that the tonsillar crypts contained large amounts of *S zooepidemicus* antigen. They speculated that infection in these horses was caused by transmission of bacteria from the tonsils to the lungs.

Colonies of *S zooepidemicus* isolated from tonsillar specimens were uniformly nonmucoid. It is tempting to speculate that upregulation of capsule expression is an important factor in opportunistic invasion by *S zooepidemicus*, because it is documented that the organism is encapsulated in smears of lung and mucopurulent material from foals with pneumonia or bronchitis.[1,5,17] In addition, Gilmour et al[18] documented that *S zooepidemicus* quickly becomes encapsulated and more virulent in the lungs of mice experimentally infected via aerosol exposure. Those authors concluded that capsule expression is important for survival and proliferation in tissue. In the study reported here, the primary cultures of specimens from 3 donkeys consisted of mucoid and nonmucoid colonies of *S zooepidemicus* that had the same SzP phenotype, suggesting rapid reversion to a nonencapsulated state. Colonies representative of these encapsulated isolates from donkeys were significantly ($P = 0.034$) more resistant to phagocytosis by neutrophils than isolates with low concentrations of cell-associated hyaluronic acid (Table 2). Although information on the characteristics of the primary colonies for the strains recovered from foals in this study were not recorded, most were nonmucoid at the time that SzP proteins and DNA were extracted, suggesting that capsule expression could have been lost after isolation. In our laboratory, colonies of *S zooepidemicus* in fresh primary cultures of lung tissue or mucopurulent material from foals with bronchopneumonia are often mucoid but rapidly lose that characteristic during subculture.

Environmental host factors and genetic mecha-

nisms that modulate hyaluronic acid synthesis in *S zooepidemicus* are poorly understood. It is possible that capsule synthesis typically is activated spontaneously at a low frequency during residence on mucosa and in the tonsils. Hypothetically, newly emerging encapsulated variants are not propagated unless the structural or functional integrity of the respiratory tract is impaired and they gain access to sites in which phagocytic cells are active, such as the submucosa, where encapsulation would provide a survival advantage. Availability of plasma components, through a substantial increase in capsule production,[16] would further enhance survivability when phagocytes are present. In addition, binding of fibrinogen to the SzP protein[17] would contribute to survivability in tissues by blocking phagocytosis. It also is possible that expression of other virulence factors, such as proteases, is coregulated with hyaluronic acid synthesis. Evidence for the possible involvement of other virulence factors include focal necrosis, degenerate leukocytes, and fibrinoid vasculitis in lungs and other tissues of horses with streptococcal pneumonia.[6]

The existence of specific strains during outbreaks of respiratory disease in young horses is not supported by the number of SzP phenotypes in foals and donkeys with pneumonia. Nevertheless, animals living as a group are more likely to host the same pool of *S zooepidemicus* strains. This was evident from the immunoblot data for the tonsillar isolates of 4 healthy horses that had been housed together for most of their lives. The same SzP immunoblot pattern was evident in isolates from each member of 2 pairs of horses. Similarly, isolates from 2 pairs of donkeys were identical for each member of the pair. Nevertheless, 3 *S zooepidemicus* strains were recognized for the influenza-induced pneumonia in the 5 donkeys, emphasizing the opportunistic nature of these infections. Finally, isolates from some of the foals with pneumonia appeared to be identical, although as a group, isolates from foals had considerable heterogeneity.

Results of SzP-specific immunoblotting, as confirmed by limited sequencing of variable sequences of SzP, is clearly a useful procedure for the study of epidemiologic characteristics and etiopathogenesis of *S zooepidemicus* infections in horses. The SzP sequences, similar to one of the Moore and Bryans serovar sequences, were recognized on all isolates of equine specimens, unlike gel-diffusion precipitating antigens that allowed identification of only 41% of isolates from equine specimens.[7] Moreover, the apparent great heterogeneity of typing antigen suggested by gel diffusion is not supported by molecular analysis. The study reported here and another study[10] in which SzP proteins of the Moore and Bryans serovars were analyzed both indicate that variability is restricted to that resulting from combination of the 2 N terminal and 5 internal HV motifs. This limits variation to 10 main variants with some additional variation contributed by differences in the number of carboxy-terminal PEPK repeats.

Additional studies should focus on the early changes in pharyngeal populations of *S zooepidemicus* in donkeys after influenza infection. Donkeys, as evident in this study and others,[2,4] are highly susceptible to secondary invasion by *S zooepidemicus* and, thus, would be excellent for use in the study of selection and emergence of *S zooepidemicus* clones expressing virulence factors, such as hyaluronic acid, during the early stages of influenza infection.

[a]Livestock Disease Diagnostic Center, University of Kentucky, 1429 Newtown Pike, Lexington, Ky.
[b]Provided by Dr. Thomas Chambers, Gluck Equine Research Center, University of Kentucky, Lexington, Ky.
[c]API, Plainview, NY.
[d]Becton Dickinson, Cockeysville, Md.
[e]Boehringer Mannheim, Indianapolis, Ind.
[f]Thermojet, Tegal Scientific, Concord, Calif.
[g]Promega, Madison, Wis.
[h]NovaBlue, Novagen, Madison, Wis.
[i]Sequenase, USB, Cleveland, Ohio.
[j]Sigma Chemical Co, St Louis, Mo.

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