Evaluation of a commercially available modified-live Streptococcus equi subsp equi vaccine in ponies

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**Objective**—To evaluate a commercially available modified-live Streptococcus equi subsp equi vaccine for safety and persistence in vaccinated ponies and to detect recombination or reversion events in the vaccine strain.

**Animals**—5 ponies that were 1.5 to 8 years old (group 1) and 4 ponies that were 6 months old (group 2).

**Procedures**—Ponies were vaccinated, with a subsequent booster vaccination 2 to 3 weeks later, and monitored for 50 days. At booster vaccination, an equal amount of a tetracycline-resistant wild-type strain of *S equi* was administered. Recovery of all strains was performed by use of bacteriologic culture and PCR assays.

**Results**—Ponies in group 1 had background antibody titers against *S equi* antigen before vaccination despite the lack of known exposure to *S equi*. Ponies in group 2 were immunologically naïve. Increases in anti-*S equi* antibody titers were detected in both groups. Ponies in group 1 did not have clinical signs of disease caused by *S equi*. In group 2, all ponies developed abscesses in retropharyngeal lymph nodes; 1 pony developed severe clinical disease and was euthanized. The vaccine strain was recovered from ponies in group 2 for up to 24 days after vaccination.

**Conclusions and Clinical Significance**—Although the vaccine was successful in inducing IgG antibodies against *S equi* in all ponies, findings suggested that the vaccine may have caused substantial morbidity and some deaths in the young ponies. In young ponies, the vaccine strain persisted in tissues for weeks; however, no evidence of recombination was detected. (Am J Vet Res 2011;72:1130–1138)
Outbreaks of strangles at breeding facilities and racetracks in the United States have become increasingly problematic, which has resulted in strict requirements by some establishments for extensive testing before movement of horses between facilities. In 2004, there was an increase in the number of samples obtained from horses with clinical signs of strangles and submitted to the University of Illinois Veterinary Diagnostic Laboratory. Many of these horses had a history of recent or prior vaccination with a commercially available modified-live S equi vaccine. Isolates of S equi with a dry colony morphology, which is characteristic of the capsular MLV strain, were recovered from these samples. Dry morphology colonies were also recovered in combination with mucoid (encapsulated) S equi colonies. It was determined by use of a 96-well microbial identification and phenotyping system that regardless of the colony morphology (dry or mucoid), several of these clinical isolates had a vaccine-like carbon source utilization phenotype. Many of these isolates also had a vaccine-like genotype as determined by means of pulsed-field gel electrophoresis. These findings, in combination with a mucoid colony phenotype in isolates cultured directly from the reconstituted vaccine, prompted the study reported here, which was a modification of a traditional safety and efficacy study for vaccine challenge exposure. The purpose of the study reported here was to test several hypotheses that attempt to explain the increase in apparent vaccine failures. These hypotheses included that the commercially available vaccine will not cause clinical signs of strangles in vaccinated ponies, will result in increased concentrations of serum anti-S equi IgG, will not be recovered at >3 days after vaccination or administration of a booster vaccination, and will not result in reversion or recombination events after vaccination.

**Materials and Methods**

**Animals**—Nine ponies (1 Haflinger, 1 Welsh, and 7 mixed-breed ponies) were procured from a local farm and transported to the University of Illinois. This farm had never had an outbreak of strangles, nor had the owner ever vaccinated any of the ponies against strangles. An attempt was made to have equal representation of both sexes; however, at the time of the study, there were more males than females, and males were subsequently overrepresented. All ponies were subjected to bacteriologic culture of nasal swab specimens and PCR testing for S equi prior to enrollment in the study. Serum titers against S equi were also measured in all ponies prior to and throughout the study. Horses were individually housed in 4.3 × 8.5-m pens. An empty pen separated adjacent horses to prevent nose-to-nose contact. Each pen was equipped with its own automatic water source and concrete feed bunk. Pens were bedded with straw, which was changed weekly. To prevent cross-contamination, animal handlers wore personal protective equipment (disposable hooded coveralls, plastic boot covers, latex gloves, a face mask, and plastic eye protection), which was changed when handlers moved between pens. All procedures were conducted in conformity with the University of Illinois Institutional Animal Care and Use Committee guidelines (protocol No. 04111) in compliance with state and federal laws and published standards.

**Experimental design**—The ponies were assigned to 2 groups. Group 1 consisted of 5 ponies (4 males and 1 female) that ranged from 1.5 to 8 years of age. Group 2 consisted of 4 ponies (3 males and 1 female) that were all approximately 6 months old.

Both groups, except for 1 pony (1.5-year-old female) in group 1 that was designated as a nonvaccinated positive control pony, were vaccinated with a modified-live S equi vaccine (day 0) and administered a booster vaccination 2 to 3 weeks later (group 1, day 17; group 2, day 22). This schedule was developed on the basis of the manufacturer’s instructions. The vaccine was reconstituted in accordance with the manufacturer’s instructions prior to inoculation. For all vaccinations and booster vaccinations, 3 mL of a suspension (1 × 10⁹ CFUs/mL) of bacteria was injected into the right nostril by use of flexible plastic syringe adapters (which resembled intrauterine catheters) provided by the vaccine manufacturer.

To assess the transfer of genetic material between wild-type S equi and the MLV strain, the wild-type challenge strain (a wild-type clinical isolate) was tagged with a tetracycline-resistance gene by mating with *Enterococcus* spp that carried transposon Tn916. The commercially available MLV strain was marked with erythromycin by use of a pCAM45 Mariner shuttle plasmid. Ponies of both groups (including the nonvaccinated control pony of group 1) were challenge exposed with equal amounts (1.3 × 10⁷ CFUs) of the tetracycline-resistance-labeled wild-type strain and erythromycin-resistance-labeled MLV strain. Thus, a 3-mL dose of a suspension (1 × 10⁷ CFUs/mL; 1:1 mixture of tetracycline-resistance-labeled wild-type strain and erythromycin-resistance-labeled MLV strain in 0.1% PBS solution) was injected into the right nostril. The interval from the booster vaccination to challenge exposure varied between groups (group 2 was challenge exposed on day 28, and group 1 was challenge exposed on day 42). In addition, on day 49, all ponies in group 1 (including the nonvaccinated control pony) received a second challenge exposure (3 mL of a 1 × 10⁹ suspension of a 1:1 mixture of equal amounts [1.5 × 10⁹ CFUs] of the tetracycline resistance–labeled wild-type strain and erythromycin resistance–labeled MLV strain, which was administered into the right nostril).

A complete physical examination, collection of nasal swab specimens and nasal washes, and endoscopy and lavage of the guttural pouch were performed on days –4, 0, 4, 8, 15, 22, 23, 28, 29, 32, 36, and 43. After the physical examination was performed, the external 0.5-cm surface of the nares and muzzle of each of the 9 ponies was cleaned with 3 X 3-inch gauze sponges soaked in a quaternary ammonium disinfectant. A sterile swab³ was inserted to a depth of approximately 6 cm into the ventral medial aspect of the nasal cavity. Care was taken to avoid the blind-ended false nostril. Swab specimens were placed in transport media and stored on ice for transport to our laboratory. Nasal washes were performed after nasal swab specimens were collected. The neck of each pony was positioned in ventroflexion,
and a sterile 60-ml catheter-tipped syringe equipped with a 10-cm extension composed of firm clear food-grade (nontoxic) sterile polyvinyl chloride tubing (0.5 cm in diameter) was used to forcefully inject 50 mL of sterile 0.1% PBS solution into the right nasal cavity. The draining effluent was immediately collected in a sterile cup and then transferred to a 50-ml sterile polypropylene centrifuge tube for transport.

Ponies were lightly sedated by IV administration of detomidine (0.01 to 0.02 mg/kg) and butorphanol tartrate (0.01 to 0.02 mg/kg) into a jugular vein. Swab specimens of the palatine tonsils then were collected from the sedated ponies of group 2 by insertion of a 1-inch, 20-gauge needle and placed into a 60-ml catheter-tip syringe. Collected lavage fluid was stored in a 50-ml sterile polypropylene centrifuge tube for transport to our laboratory. To prevent cross-contamination, the endoscope was thoroughly disinfected with a 3.2% glutaraldehyde solution for a minimum of 15 minutes between successive uses in different ponies. After disinfection with the glutaraldehyde solution, the scope and injection ports were repeatedly rinsed with sterile saline (0.9% NaCl) solution, and a 25-ml sample of the final rinse with saline solution (endoscope control wash) was collected in a 50-ml sterile polypropylene centrifuge tube for bacteriologic culture and PCR assay.

After completion of the guttural pouch lavage, samples were obtained from the submandibular lymph nodes. Nodes were gently aspirated by use of a 21-gauge, 1-inch needle attached to a 3-ml syringe.

In addition, ponies were evaluated daily for signs of disease or distress as assessed on the basis of several criteria and a score was assigned to represent the severity of clinical disease (Appendix). Any pony with a score > 9 was immediately euthanized.

Blood samples were collected from a jugular vein by use of a 1-inch, 20-gauge needle and placed into EDTA-containing tubes and plain glass tubes on days 0, 8, 15, 21, 28, and 36 and immediately before horses were euthanized at the end of the study. Samples were used for serologic evaluation, CBCs, and serum biochemical analyses.

All surviving ponies were euthanized at the end of the study (days 43 to 50 for ponies in group 2 and day 57 for ponies in group 1). Ponies were euthanized by administration of an overdose of barbiturate euthanasia solution. Necropsies were performed on all ponies.

**Bacteriologic culture**—All collected samples were submitted for bacteriologic culture on Columbia blood (5% sheep blood) agar, Columbia colistin and nalidixic acid agar, and brain-heart infusion broth plates that contained 5% sheep blood agar were supplemented with 64 µg of amikacin/mL. Naturally developing resistance to amikacin was used to recover all strains of *S equi* and suppress overgrowth of other bacteria. For fluid samples, 100-µL aliquots were plated for quantitative bacteriologic culture. All plates were incubated overnight at 37°C in 5% CO₂. An enrichment step was used on all samples to increase the sensitivity of the recovery of streptococci. After initial plating, the swab tips and 100-µL aliquots of fluid samples were placed in 9 mL of thioglycollate broth with dextran supplement and subcultured on plates containing purple broth with 1.25 g of lactose/mL. Metabolism of lactose by strains of *Streptococcus equi* subsp *zooepidemicus* was evident as a decrease in pH indicated by the yellow color of the agar (positive result), and strains of *S equi* were identified by their inability to use lactose, which resulted in maintenance of the purple color of the agar (negative result). Final identification of representative streptococcal isolates was confirmed by use of a 96-well microbial identification and phenotyping system.

**S equi–specific PCR assay**—The DNA was isolated by use of a DNA extraction kit used in accordance with the manufacturer's directions for preparation of tissue samples. Briefly, each nasal and tonsil swab specimen was placed separately in a 1.5-ml microcentrifuge tube that contained 1 mL of sterile saline solution; tubes were vortexed and then centrifuged for 3 minutes at approximately 8,000 × g. Similarly, 1 mL of fluid from nasal washes and the endoscope control washes was placed in similar tubes and centrifuged at approximately 8,000 × g for 3 minutes. In accordance with the manufacturer's directions for modifications for tissue samples and a final elution volume of 100 µL, DNA was isolated from the resulting pellets. Purified DNA was subjected to a multiplex PCR assay with *S equi*–specific primers that amplified a 680-bp region of the M-protein gene *SeM* (Sem 680; forward primer, 5′-TGCAAAAAGAGTTCTCCTGTC-3; reverse primer, 5′-GATTGGTGAAGAGTTGAGC-3′) and universal primers that amplified the bacterial 16S ribosomal (*rrs*) gene (*rrs*; forward primer, 5′-GAGATTTACACCTGTGTAAC-3; reverse primer, 5′-TGTTTGCGGACTTACCACAC-3′). Amplification of the *rrs* gene was used as a positive internal control test to ensure sample quality, and results for samples that failed to amplify the *rrs* gene were not interpreted. The PCR amplification of
the 5′-end and 3′-end genes was performed by use of a 10% reaction mixture that contained 183 μL of distilled water, 30 μL of 10X buffer, 6 μL of deoxyribonucleoside triphosphate mix (10mM), 3 μL of each primer (25μM), 24 μL of MgCl2 (25mM), and 1 μL of DNA polymerase (5 U/μL). An aliquot (5 μL) of template was used in a 30-μL reaction. The PCR amplification was performed by use of a thermal cycler with the following settings: 95°C for 2 minutes; 40 cycles of 95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes; and holding at 4°C. The PCR products were assayed on 1% agarose gels, stained with ethidium bromide, and transilluminated with UV light.

Fine-needle aspirates of submandibular lymph nodes—Aspirates obtained from the submandibular lymph nodes were placed on glass slides and stained (Wright-Giemsa stain) by use of an automated device. Slides were evaluated by a board-certified veterinary clinical pathologist (AMB). Slides were categorized as normal lymph node, reactive lymph node, or suppurrative lymphadenitis.

ELISA—A whole-cell antigen ELISA was used to monitor serologic response to vaccination and challenge exposure. Briefly, each well of 96-well plates was coated with a 0.5 McFarland solution (200 μL/well) of the mucoid or dry phenotype of the vaccine strain suspended in coating buffer (13.56 g of Na2CO3, 22.84 g of NaHCO3, and 0.813 g of MgCl2 in 4 L of distilled water [pH, 9.6]); plates were incubated at 22°C for 1 hour. Mucoid and dry phenotypes were used because the mucoid hyaluronic acid capsule potentially could have altered the array of detectable surface epitopes. After incubation, plates were washed 3 times with washing buffer (36 g of saline solution and 1.83 mL of Tween 20 in 4 L of distilled water) and blocked by the addition of 200 μL of 0.01% bovine serum albumin in diluent buffer (35.06 g of saline solution, 1.6 mL of Tween 20, 1.83 g of NaH2PO4, and 7.19 g of NaHPO4 in 4 L of distilled water [pH, 7.1]) and incubation for 2 hours at 22°C. The plates then were washed 3 times with washing buffer. For serum IgG measurements, pony sera were diluted 1:200, 1:400, 1:800, 1:1,600, and 1:3,200 in diluent buffer, and 200 μL of diluted serum was added to the appropriate wells. The plates were incubated for 1 hour at 22°C and then washed 3 times with washing buffer. Goat anti-equine IgG conjugated to alkaline phosphatase reporter was diluted 1:100 in diluent buffer, and 200 μL of the diluted solution was added to the appropriate wells. The plates again were incubated for 1 hour at 22°C and subsequently washed 3 times with washing buffer. Fresh substrate (p-nitrophenyl phosphate tablets diluted to 1 mg/mL in substrate diluent [9.3 g of Na2CO3, 9.41 g of NaHCO3, and 0.813 g of MgCl2 in 4 L of distilled water [pH, 9.8]]) was added to the appropriate wells in 200-μL aliquots. The plates were incubated at 22°C for 30 minutes, and absorbance was measured at 405 nm. Each plate included duplicate control wells (no antigen, no serum sample, no secondary antibody, no substrate, no blocking solution, and blank control samples). Each plate also contained a positive control sample (serum from a horse with a known titer against S equi) in triplicate. The absorbance of all wells was standardized and expressed as a percentage of the positive control sample.

Statistical analysis—For the ELISA data, a Student t test was used to compare mean absorbances among ponies and between groups by use of commercially available software. For all tests, values of P < 0.05 were considered significant.

Results

Physical examination, hematologic evaluation, bacteriologic culture, and PCR assay—Ponies in group 1, including the nonvaccinated challenge-exposed control pony, did not have any values outside the reference limits for any of the measured clinical or hematologic variables. Results of bacteriologic cultures of nasal swab specimens, nasal washes, and guttural pouch lavages were negative throughout the experiment, except for samples obtained on day 50 (ie, 1 day after the second challenge exposure). Both the erythromycin resistance–labeled MLV strain and tetracycline resistance–labeled wild-type strain were recovered. The MLV strain colonies with the mucoid phenotype were recovered more frequently (1, 25, and 37 CFUs in the nasal washes of 3 ponies; rare growth in the nasal swab specimen of 1 pony; and light growth in the nasal swab specimen of the nonvaccinated control pony) than were colonies with the dry phenotype (1 CFU in a nasal wash of 1 pony). The tetracycline resistance–labeled wild-type strain was cultured on day 50 from nasal washes (5, 10, 12, and > 250 CFUs in samples obtained from the 4 ponies and 5 CFUs in the sample obtained from the nonvaccinated control pony) and nasal swab specimens (rare growth in samples obtained from 1 pony and light growth in samples obtained from 3 ponies (one of which was the nonvaccinated control pony). Although the recovery of S equi isolates was generally low, more S equi isolates were recovered from nasal washes than from nasal swab specimens or guttural pouch lavages (none of the guttural pouch lavages yielded S equi isolates).

Results of PCR assay for the 5′-end gene were negative for all samples throughout the experiment, except for 1 weak positive result that was detected in an S equi isolate cultured in a nasal wash obtained on day 50. Sample inhibition was a recurring problem, with failure to amplify the 5′-end gene in many samples. Because of the frequent PCR inhibition, bacteriologic culture was a more sensitive method for detection of S equi. The use of selective agar and broth greatly increased sensitivity.

In contrast to results for group 1, ponies in group 2 had changes in several clinical variables (including nasal discharge, lethargy, and mild fever) after initial vaccination. Two of the ponies developed a mild fever on day 15 that coincided with rupture of retropharyngeal lymph node abscesses into the guttural pouches (Figure 1). Two additional ponies had spikes in rectal temperature that coincided with rupture of lymph node abscesses on days 23 and 28 (second bout of lymph node rupture). The findings were observed following the initial and booster vaccinations, but the ponies (except for 1 pony) improved after challenge exposure.
After the initial vaccination, the retropharyngeal lymph nodes of all ponies in group 2 became mildly swollen. This finding was difficult to appreciate during physical examination in 3 ponies, but prominent swelling of the lymph nodes could be seen during endoscopy of the guttural pouch (Figure 2). This swelling progressed to formation of abscesses and rupture of the retropharyngeal lymph nodes of the 3 ponies on day 15, which corresponded with a spike in rectal temperature on day 15 in 2 of the ponies. One pony had palpably swollen retropharyngeal lymph nodes after initial vaccination that also were visible during endoscopy of the guttural pouch; however, rupture of the lymph nodes into the guttural pouch in this pony was much later (day 28).

Clinical scores were assigned for the swelling, abscess formation, and rupture of retropharyngeal lymph nodes. For the 4 ponies in group 2, median clinical scores on days 15, 22, 23, 25, 28, 29, and 32 were 3 (range, 1 to 3), 1 (range, 1 to 2), 2 (range, 2), 1.5 (range, 1 to 2), 1 (range, 1 to 3), 1.5 (range, 1 to 2), and 1 (range, 1 to 2), respectively. On day 36, the clinical score was not determined for 1 pony and the median for the remaining 3 ponies was 1 (range, 1 to 2). One pony was euthanized on day 36. On day 43, 2 of the remaining ponies had a clinical score of 0 and the other pony had a clinical score of 1. There was an increase in clinical score in the days following rupture (day 15 for 3 ponies and day 28 for the other pony). One of the 3 ponies that had rupture of the lymph nodes on day 15 had lymph nodes that were periodically swollen, which was accompanied by mucopurulent discharge. Interestingly, this pony had long-term tonsilar carriage (46 days) of the vaccine strain (data not shown).

Over the course of the study, one of the ponies in group 2 developed progressive lethargy, fever, and dyspnea with inspiratory stridor and was euthanized on day 36. The clinical signs of respiratory distress were particularly obvious when the pony was being examined or during collection of samples. Physical examination revealed that the cervical and retropharyngeal lymph nodes were progressively enlarging, warm, and firm to fluctuant and were causing the pony signs of pain. In addition, this pony developed progressive leukocytosis (WBC count on days –4, 25, and 36 was 13,500, 23,300, and 31,700 cells/µL, respectively) with mature neutrophilia (neutrophil count on days –4, 25, and 36 was 5,940, 11,700, and 26,100 cells/µL, respectively) and an elevated fibrinogen concentration (fibrinogen concentration on days –4, 25, and 36 was 200, 900, and 700 mg/dL, respectively), all of which were consistent with inflammation. Although fine-needle aspirates were not routinely obtained from ponies in group 2 because of the low yield for ponies in group 1, aspirates of lymph nodes were obtained from this pony because of the severe lymph node enlargement. Analysis of fine-needle aspirates of submandibular lymph nodes obtained from this pony revealed suppurative lymphadenitis with intracellular cocci.

Dry and mucoid phenotypes of the vaccine strain were detected in approximately equal numbers and most frequently following rupture of the retropharyngeal lymph nodes on day 15 in 3 ponies and day 28 in the other pony of group 2 (Figure 3). The MLV strain could be recovered from the guttural pouch of the pony without clinical signs up to 25 days after initial vaccination, from nasal swab specimens obtained from another pony for up to 36 days after vaccination, and from the tonsil swab specimen of a third pony for up to 46 days after vaccination. The MLV strain was recovered from guttural pouch lavages and nasal washes more frequently and in much greater numbers than from nasal or tonsil swab specimens. All ponies (except for 1, which was euthanized because of severe clinical disease following vaccination) did not develop additional clinical signs. The PCR assay was not attempted for samples obtained from group 2 because of the results for group 1, whereby bacteriologic culture was found to be the more sensitive method for detection because PCR inhibition was common.

Cytologic examination of fine-needle aspirates of submandibular lymph nodes—Fine-needle aspirates of submandibular lymph nodes were classified as indicative of normal or reactive lymph nodes in all ponies throughout the study. No evidence of suppurative lymphadenitis was detected.

ELISA—Increases in serum IgG titers against S equi were detected following vaccination and challenge exposure, with high-
er titers when the mucoid phenotype of the MLV strain was the plate antigen (Figure 4). Interestingly, 1 pony in group 1 had a delayed and limited humoral response, which was significantly \( P = 0.02, P = 0.036, \) and \( P = 0.006 \) lower than that for the 3 other vaccinated ponies but was not significantly \( P = 0.83 \) different from that for the nonvaccinated control pony, as determined by use of ELISA data for the mucoid phenotype of the MLV strain. This pony was heavily colonized with \( S \) equi subsp zooepidemicus, which was routinely recovered in large numbers from the tonsils, nasal cavity, and guttural pouches. It is possible that competition for colonization between the MLV strain and the resident \( S \) equi subsp zooepidemicus may have decreased antigen activity and, in turn, the immune response of this pony.

In contrast to group 1, group 2 had significantly lower background antibody titers against the dry \( P = 0.032 \) and mucoid \( P = 0.019 \) phenotype whole-cell \( S \) equi antigen. A moderate increase in serum IgG titers against \( S \) equi was detected following vaccination and administration of a booster vaccination. Serum antibody concentrations peaked between days 25 and 36 in 3 ponies (Figure 5). One clinically ill pony had a steady increase in circulating antibody concentrations until it was euthanized on day 36.

Necropsy findings—No lesions were detected in any ponies in group 1. No \( S \) equi strains were grown on bacteriologic culture of multiple tissues, including the retropharyngeal lymph nodes, guttural pouch mucosa, tracheobronchial lymph nodes, lungs, trachea, and spleen.

Gross and histologic postmortem findings were unremarkable in 3 ponies in group 2; however, findings characteristic of disseminated (ie, bastard) streples were observed in the pony that was euthanized because of severe clinical disease. Physical examination revealed that this pony had a mild to moderate amount of nasal discharge, and a 20 X 10 X 10-cm firm mass, which extended cranially from the thoracic inlet, was palpable on the left lateral aspect of the neck. The subcutaneous tissues overlying and distal to the mass were clear, light yellow, thickened, and edematous. The mass consisted of approximately 4 to 6 multifocal to coalescing, thickly encapsulated nodules that ranged in size from 10 X 6 X 6 cm to 3 X 2 X 2 cm; incision of these nodules released a creamy yellow-to-green exudate (approx 100 mL of exudate was collected from all the nodules). The mass impinged on the trachea and displaced it dorsally. Examination of Gram-stained slides of the exudate revealed many chains of gram-positive cocci among inflammatory cells, which included degenerative neutrophils and macrophages. The retropharyngeal lymph nodes were similarly enlarged bilaterally (4 X 3 X 3 cm), and each contained approximately 1 mL of creamy yellow-to-green exudate. The right retropharyngeal lymph node had ruptured, and the exudate was in the right guttural pouch. The mediastinal and tracheobronchial lymph nodes were enlarged (3 X 2 X 2 cm), edematous, and
The submandibular lymph nodes were not grossly enlarged, and no other changes were observed. The cause of the respiratory distress in this pony was most likely compression and obstruction of the trachea by the large mass at the thoracic inlet. The mass appeared to consist of a chain of enlarged and abscessed lymph nodes.

Bacteriologic culture confirmed the presence of dry and mucoid phenotypes of the \textit{S. equi} MLV strain. The same numbers of colonies for both the dry and mucoid phenotypes were cultured from the exudate (250 CFUs), neck abscesses (250 CFUs), retropharyngeal lymph nodes (10 CFUs), and tracheobronchial lymph nodes (10 CFUs); the dry phenotype (3 CFUs) also was cultured from the mediastinal lymph nodes. The resistance patterns of the isolated organisms suggested that these isolates were from the initial vaccination and booster vaccination (ie, unaltered vaccine strain). No erythromycin resistance–labeled MLV strain or tetracycline resistance–labeled wild-type strain was cultured. However, \textit{S. equi} subsp \textit{zooepidemicus} organisms were cultured from the guttural pouches (50 CFUs), nasal mucosa (50 CFUs), retropharyngeal lymph nodes (5 CFUs), tonsils (10 CFUs), and tracheobronchial lymph nodes (5 CFUs).

Histologic examination of the abcesses from this pony revealed lymph node tissue associated with the mass in the ventral aspect of the neck (Figure 6). The large, lobulated mass was most likely a chain of abscessed lymph nodes. Left and right retropharyngeal as well as cervical lymph nodes (associated with the lesion) contained many neutrophils, pockets of degenerate neutrophils, and macrophages surrounded by a thick fibrous capsule. The amount of fibrosis and number of macrophages within the exudate suggested a chronic but active lesion. Nonabscessed lymph nodes appeared indistinguishable from those harvested from the other ponies.

**Genetic exchange in vivo**—No in vivo exchange of antimicrobial-resistance genes was detected in any recovered strain of \textit{S. equi}; however, during the course of the study, 6 isolates of tetracycline-resistant \textit{S. equi} subsp \textit{zooepidemicus} were recovered following challenge exposure. The PCR amplification of the \textit{S. equi} subsp \textit{zooepidemicus} strain with the tetracycline-resistance gene confirmed the transposition of Tn916 into the genome.
that these titers (often measured against *S. equi* subsp. *zooepidemicus*) did not appear to be safe for use in young ponies. All ponies in this age group developed abscesses in the retropharyngeal lymph nodes, which likely would not have been detected had endoscopic examinations of the guttural pouch not been routinely performed. In addition, 1 pony developed severe clinical signs of strangles that resulted in a decision to euthanize that pony. Although that pony was challenge exposed with a wild-type strain, the bacterial strains recovered from abscessed lymph nodes and exudate were consistent with the unaltered MLV strain, and no isolates of tetracycline resistance–labeled wild-type strain were found. The dry and mucoid phenotypes of the MLV strain were recovered in nearly equal proportions, which was unexpected because theoretically the mucoid variant should have a survival advantage. The hyaluronic acid capsule is a virulence factor and is important for evading host defenses. As such, greater numbers of mucoid colonies than dry colonies were expected within the exudate.

It is unclear whether age or immune status was the determining factor in morbidity and death associated with the MLV strain. Additional studies with ponies that are older but still immunologically naïve would be beneficial; however, several researchers have found it difficult to locate ponies or horses that do not have background titers against *S. equi*. Mucoid movement of genetic material into other species is recognized, and current recommendations for the detection of carrier animals are bacteriologic culture or PCR assay of guttural pouch swabs. It is probable that similar abscesses and rupture into the guttural pouches occur in naïve vaccinated horses in on-farm settings. In fact, the delayed rupture of retropharyngeal lymph nodes in one of the ponies in the present study may have been responsible for the increased morbidity observed in that pony. *Streptococcus equi* subsp. *equi* persists in the guttural pouches and often is incorporated into hard concretions of inspissated pus (ie, chondroids).

Persistence of the MLV strain is a concern because the potential for recombination or reversion of the MLV strain increases if the organism is not eliminated by the host. Spread of the MLV strain via vaccinated carrier animals to naïve populations, nonvaccinated horses (such as those in a boarding facility), or farms free of strangles could be problematic. It is theoretically possible for attenuated strains to revert to virulence via the exchange of genetic material or spontaneous reversion. Tetra-cycline-resistant strains of *S equi* subsp *zooepidemicus* carrying Tn916 were recovered from ponies following challenge exposure. This finding supports the potential for genetic exchange between these closely related organisms. However, there was no evidence of the movement of genetic material into other *S equi*. Mucoid revertants of the MLV strain were easily recovered in vitro and in vivo. This is problematic because the lack of a capsule is often cited as an important mechanism of attenuation of this strain; however, the actual molecular mechanism of attenuation in this strain is not definitively known.

It is important to mention that the MLV could safely be administered to adult ponies with low background antibody titers against *S equi*. Administration of

**Discussion**

The study reported here highlighted the incomplete attenuation of the currently available MLV strain, which did not appear to be safe for use in young (<1-year-old) naïve ponies. All ponies in this age group developed abscesses in the retropharyngeal lymph nodes, which likely would not have been detected had endoscopic examinations of the guttural pouch not been routinely performed. In addition, 1 pony developed severe clinical signs of strangles that resulted in a decision to euthanize that pony. Although that pony was challenge exposed with a wild-type strain, the bacterial strains recovered from abscessed lymph nodes and exudate were consistent with the unaltered MLV strain, and no isolates of tetracycline resistance–labeled wild-type strain were found. The dry and mucoid phenotypes of the MLV strain were recovered in nearly equal proportions, which was unexpected because theoretically the mucoid variant should have a survival advantage. The hyaluronic acid capsule is a virulence factor and is important for evading host defenses. As such, greater numbers of mucoid colonies than dry colonies were expected within the exudate.

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Persistence of the MLV strain is a concern because the potential for recombination or reversion of the MLV strain increases if the organism is not eliminated by the host. Spread of the MLV strain via vaccinated carrier animals to naïve populations, nonvaccinated horses (such as those in a boarding facility), or farms free of strangles could be problematic. It is theoretically possible for attenuated strains to revert to virulence via the exchange of genetic material or spontaneous reversion. Tetra-cycline-resistant strains of *S equi* subsp *zooepidemicus* carrying Tn916 were recovered from ponies following challenge exposure. This finding supports the potential for genetic exchange between these closely related organisms. However, there was no evidence of the movement of genetic material into other *S equi*. Mucoid revertants of the MLV strain were easily recovered in vitro and in vivo. This is problematic because the lack of a capsule is often cited as an important mechanism of attenuation of this strain; however, the actual molecular mechanism of attenuation in this strain is not definitively known.

It is important to mention that the MLV could safely be administered to adult ponies with low background antibody titers against *S equi*. Administration of
initial and booster vaccinations to these ponies resulted in increased antibody titers. A major limitation of the study reported here was the inability to induce clinical disease in the nonvaccinated control pony. The challenge strain was recovered from a sample obtained from a clinically affected horse with lymph node abscesses; as such, it was assumed to be virulent. It is possible that the challenge strain was inadvertently attenuated during the genetic manipulations required to insert a tetracycline-resistance gene. Serial passage may have induced partial attenuation of this strain. Alternatively, the nonvaccinated control pony had a high background antibody titer against whole-cell S. equi antigen, which may have been immunologically protective against the challenge strain. In either case, because we could not induce clinical disease with the challenge strain, we cannot accurately interpret the findings regarding the efficacy of vaccination in this study.

References

Appendix
Criteria used to assign a clinical score for the severity of clinical disease caused by *Streptococcus equi* susp. *equi* in ponies.*

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal temperature (°C)</td>
<td>≤ 38.0</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>Mild enlargement</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>Slight or serous</td>
</tr>
<tr>
<td>Hematologic analysis</td>
<td>Within reference limits for a stress leukogram</td>
</tr>
<tr>
<td>Endoscopy of the ventral diverticulum of the auditory tube (guttural pouch)</td>
<td>Mild swelling of retropharyngeal lymph nodes</td>
</tr>
<tr>
<td>Miscellaneous clinical signs (anorexia, coughing, neck extended, dyspnea, dysphagia, and increase in lung sounds)</td>
<td>1 sign</td>
</tr>
</tbody>
</table>

*In the present study, any pony with a score ≥ 9 was euthanized immediately.*

References
c. BD Diagnostics, Franklin Lakes, NJ.d. Roccal-D, Sigma-Aldrich Co, St Louis, Mo.
e. Corning Inc, Corning, NY.
g. Advanced Sterilization Products, Irvine, Calif.
h. Remel, Lenexa, Kan.i. Qiagen, Valencia, Calif.
j. Applied Biosystems, Foster City, Calif.
k. Global Medical Instrumentation Inc, Ramsey, Minn.l. Hema-tek, Mishawaka, Ind.