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)

Bacterial disease in horses caused by Streptococcus equi subsp equi (a Lancefield group C streptococcus and the agent of strangles) is evident throughout the world. It is highly contagious and was first recognized in medieval Europe. The organisms infect the oropharynx and adjacent lymph nodes of equids. The disease progresses with fever, swelling of local lymph nodes, and formation of abscesses and purulent nasal discharge. Typical hematologic abnormalities in horses with clinical strangles include leukocytosis (approx 30,000 cells/µL) with a neutrophil count 25,000 cells/µL and a plasma fibrinogen concentration >600 mg/dL. Mature abscesses rupture externally through the overlying skin and subcutis or internally into the ventral diverticulum of the auditory tube (guttural pouch). Compression of the trachea by swollen lymph nodes can cause respiratory distress, and the name strangles derives from recognition of this clinical sign. Although strangles is usually limited to the retropharyngeal or submandibular lymph nodes, it can spread (presumably via the hematogenous route) to distant sites, including the lungs, tracheobronchial lymph nodes, mesenteric lymph nodes, muscles, and brain. Convalescent horses may continue to harbor bacteria in the guttural pouch and may act as a reservoir of the disease. Because S equi is fragile in the environment, the development of carrier animals is fundamental to the survival of the bacteria between outbreaks. Strangles is often accompanied by loss of condition with the potential for visible scarring, and serious complications can arise in 20% of affected animals, with a case fatality rate of approximately 8%. The economic impact of this disease on the equine industry is profound because of the high prevalence of disease, with estimates of approximately 30% of infections in horses throughout the world attributable to S equi.
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 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 vaccines 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 × 10⁷ 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 to 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 (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 × 10⁸ suspension of a 1:1 mixture of equal amounts [1.5 × 10⁷ 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 gullet 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 nare and muzzle of each of the 9 ponies was cleaned with 3 × 3-inch gauze sponges soaked in a quaternary ammonium disinfectant. A sterile swab 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 guarded uterine swab into the oral cavity. Endoscopy of the guttural pouch was performed in each of the 9 sedated ponies. A biopsy probe was used to guide the endoscope into the guttural pouch. Both guttural pouches were evaluated. Approximately 40 mL of sterile PBS solution was used to lavage the right guttural pouch, which was then aspirated through the biopsy port of the endoscope by use of 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 6.8 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 dextrose supplemented with 6.8 µg of amikacin/mL. Plates then were incubated for 24 hours at 37°C and 5% CO₂, after which 100 µL of the enrichment broth was used for bacteriologic culture on selective and nonselective media as described previously. Samples collected after inoculation of ponies with antimicrobial-marked strains were submitted for bacteriologic culture on additional selective media (brain-heart infusion broth plates with 5% horse blood agar supplemented with 1 µg of erythromycin/mL, 5 µg of tetracycline/mL, or both).

All plates were evaluated for β-hemolytic colonies characteristic of streptococci. Numbers of CFUs for samples of nasal washes and guttural pouch lavages were counted. For swab specimens, semiquantitative growth estimates were reported as rare, light, moderate, or heavy (growth confined to the primary quadrant was recorded as rare; growth extending into the second, third, and fourth quadrants was reported as light, moderate, and heavy, respectively). Isolates were further evaluated by use of Gram stain and 0.3% hydrogen peroxide. Gram-positive, catalase-negative cocci were subcultured on plates containing purple broth supplemented 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’-TGCATAAAAGAAGTTCTGTC-3’; reverse primer, 5’-GATGGTGTAAGAGCTTGAGC-3’), and universal primers that amplified the bacterial 16S ribosomal (rrs) gene (rrs; forward primer, 5’-GGATATGATACCCCTGGTAGTC-3’; reverse primer, 5’-TGTTGCGGGACTTAAACCAAC-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 SemM and rrs 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 polymerase1 (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 cycler7 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 NaHPO4, and 0.8 g of NaN3 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 1.905 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 NaHPO4, 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 reporter7 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 tablets7 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 SemM 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 rrs 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 13 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 13,300, 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-

Figure 1—Rectal temperatures of four 6-month-old ponies (group 2) that received an initial vaccination with a commercially available modified-live Streptococcus equi vaccine (day 0) and a booster vaccination on day 22 and were challenge exposed with a tetracycline resistance–labeled wild-type strain and an erythromycin resistance–labeled MLV strain on day 28. Each symbol represents results for 1 pony. Notice the increase in rectal temperature on day 15 and 23 in most ponies, which coincided with rupture of abscesses of the retropharyngeal lymph nodes into a ventral diverticulum of the auditory tube (guttural pouch). Also notice the dramatic increase in rectal temperature of 1 pony that coincided with an increase in the clinical signs; that pony was euthanized on day 36.
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) streptococcal abscesses 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 × 10 × 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 × 6 × 6 cm to 3 × 2 × 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 × 3 × 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 × 2 × 2 cm), edematous, and

Figure 2—Endoscopic images of the guttural pouches of a representative pony of group 2 depicting inflammation and swelling of the retropharyngeal lymph nodes (arrowhead; A), exudate at the entrance to the guttural pouch (long arrow; B), and rupture of the retropharyngeal lymph node with exudate (short arrow; C).

Figure 3—Bacteriologic culture results for guttural pouch lavage samples obtained on various days during the study and at necropsy (Nec) from the 4 ponies of group 2. Each bar pattern represents results for 1 pony. Dry and mucoid colony phenotypes of the unmarked MLV strain were detected most frequently following rupture of the retropharyngeal lymph node abscesses on day 15 in 3 ponies and on day 28 in the other pony. The MLV strain was recovered from the guttural pouches of 1 pony that did not have clinical signs for up to 25 days after initial vaccination.
reddened. 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 *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 and the weak immune response of one of the ponies. Results are reported as the absorbance (measured at 405 nm) as a percentage of the absorbance for a positive control sample.

Histologic examination of the abscesses 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 *S equi*; however, during the course of the study, 6 isolates of tetracycline-resistant *S equi* subsp *zooepidemicus* were recovered following challenge exposure. The PCR amplification of the *S equi* subsp *zooepidemicus* strain with the tetracycline-resistance gene confirmed the transposition of Tn916 into the genome.
tulated10,11 that these titers (often measured against AJVR, Vol 72, No. 8, August 2011 1137 which did not appear to be safe for use in young Discussion

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area of neutrophils and necrotic debris (arrow). H&E stain; bar = 500 μm.

Figure 6—Photomicrograph of a tissue section obtained from a large cervical mass of a pony; the mass was a chain of abscessed lymph nodes. Notice the rim of lymphoid tissue (arrowhead) consistent with the remnant of a lymph node adjacent to the large area of neutrophils and necrotic debris (arrow). H&E stain; bar = 500 μm.

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 strangulation 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. Administration of these titers may represent nonspecific cross-reactive antibodies developed against S. equi subsp. zooepidemicus or other commensal streptococci. In either case, the background titers in group 1 were significantly higher than those in group 2 and serologic responses typically were more rapid in group 1 than in group 2, which suggested an anamnestic response. Also of interest was the unexpectedly weak serologic response of 1 pony in group 1. Although host factors could have been the underlying cause, further examination of the bacteriologic culture findings for this pony revealed high numbers of S. equi subsp. zooepidemicus, which were recovered frequently from nasal washes, swab specimens, and guttural pouch lavages. This strain of S. equi subsp. zooepidemicus produced a bacteriocin-like inhibitory substance that was bactericidal to all isolates of S. equi used in the present study. This putative bacteriocin-like inhibitory substance is an approximately 2-kDa lipoprotein and is unique from a previously identified bacteriocin-like inhibitory substance of S. equi subsp. zooepidemicus (zoocin A). Purification and characterization of this small peptide are in progress.

Another important finding was the persistence of the MLV strain for much longer than expected. The MLV strain was recovered for up to 46 days after initial vaccination (24 days after administration of the booster vaccination). Bacteriologic culture of the MLV strain was correlated temporally with lesions in the guttural pouches, and in many cases, S. equi was only recovered from the guttural pouches. Carriage of S. equi within the guttural pouches is recognized, and current recommendations for the detection of carrier animals are bacteriologic culture or PCR assay of guttural pouch washes. 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. Tetacycline-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.

Criteria used to assign a clinical score for the severity of clinical disease caused by Streptococcus equi

**Appendix**

<table>
<thead>
<tr>
<th>Criterion</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal temperature (°C)</td>
<td>≤ 38.0</td>
<td>38.0–39.4</td>
<td>≥ 39.5</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>Mild enlargement</td>
<td>Mild enlargement; warm or causes signs of pain</td>
<td>Mild enlargement; warm or causes signs of pain; abscess formation (with or without rupture)</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>Slight or serous</td>
<td>Moderate suppurative</td>
<td>Copious suppurative</td>
</tr>
<tr>
<td>Hematologic analysis</td>
<td>Within reference limits for a stress leukogram</td>
<td>Neutrophilia; plasma fibrinogen concentration ≤ 600 mg/dL</td>
<td>≥ 30,000 WBCs/mL or ≥ 25,000 neutrophils/mL; plasma fibrinogen concentration &gt; 600 mg/dL</td>
</tr>
<tr>
<td>Endoscopy of the ventral diverticulum of the auditory tube (guttural pouch)</td>
<td>Mild swelling of retropharyngeal lymph nodes</td>
<td>Moderate swelling of retropharyngeal lymph nodes or mucopurulent discharge</td>
<td>Abscesses in retropharyngeal lymph nodes or copious purulent discharge</td>
</tr>
<tr>
<td>Miscellaneous clinical signs (anorexia, coughing, neck extended, dyspnea, dysphagia, and increase in lung sounds)</td>
<td>1 sign</td>
<td>2 signs</td>
<td>≥ 3 signs</td>
</tr>
</tbody>
</table>

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

References