Evaluation of 5 serologic assays to detect *Rhodococcus equi* pneumonia in foals

Ronald J. Martens, DVM; Noah D. Cohen, VMD, MPH, PhD, DACVIM; M. Keith Chaffin, DVM, MS, DACVIM; Shinji Takai, DVM, PhD; Charity L. Doherty, BS; Arthur B. Angulo, DVM, PhD; Ronnie F. Edwards, DVM

Objective—To determine the sensitivity and specificity of 5 serologic assays used to diagnose *Rhodococcus equi* pneumonia in foals and to determine whether any of the assays could be used to identify affected foals prior to the onset of clinical signs or to differentiate between affected and unaffected foals when clinical signs first become apparent.

Design—Nested case-control study.

Animals—26 foals.

Procedure—Serum samples were obtained from all foals at 2, 4, and 6 or 7 weeks of age. Additional samples were obtained from affected foals at the time of diagnosis of *R equi* pneumonia and from age-matched unaffected foals. Samples were tested with 3 ELISA, an agar gel immunodiffusion assay, and a synergistic hemolysis inhibition assay.

Results—Sensitivity and specificity data indicated that none of the assays could be used to reliably differentiate affected from unaffected foals at any testing period. Proportions of foals that had an increase in test values between paired samples collected at 4 and 6 or 7 weeks of age were not significantly different between affected and unaffected foals. For all assays, result values increased significantly over time; however, the rate of increase was not significantly different between affected and unaffected foals.

Conclusions and Clinical Relevance—Results suggest that serologic assays, whether performed on single or paired samples, cannot be used to reliably establish, confirm, or exclude a diagnosis of *R equi* pneumonia in foals. (*J Am Vet Med Assoc* 2002;221:825–833)

Rhodococcus equi is a gram-positive, facultative-intracellular, soil-borne bacterium that causes severe pneumonia in foals and immunocompromised humans and has a world-wide distribution.[1] Inhalation of bacteria-laden dust is thought to be the most common route of exposure and infection in foals.[1,2] Epidemiologic evidence indicates that foals that develop *R equi* pneumonia are most commonly infected during the first few days of life,[3] but clinical signs typically do not develop until foals are 30 to 60 days old and may not be apparent for several months.[3,4]

Only *R equi* strains that have an 85- to 90-kilobase virulence-associated plasmid are considered capable of causing disease in foals.[5] These plasmids and their 15- to 17-kd **virulence-associated protein antigen (VapA)** products and encoding genes represent markers of virulence, but specific virulence factors have not been identified.[5,6] However, the widespread distribution of this soil-borne organism and published serologic evidence that most horses develop *R equi*-specific antibodies[1,2,5,7] suggest that most horses are exposed to virulent or avirulent strains of the organism, or both.

Intravenous administration of *R equi* hyperimmune plasma can prevent the ravaging effects of *R equi* pneumonia; however, its use is expensive and labor-intensive, and treatment with hyperimmune plasma is not universally effective.[8-12] Because of these limitations and the lack of an effective vaccine, antimicrobial treatment is currently the key to managing *R equi* infections in foals. Thus, highly accurate diagnostic tests that consistently detect *R equi* infection early in the course of disease, preferably prior to the onset of clinical signs, would be highly desirable. *Rhodococcus equi* pneumonia is oftentimes life or career threatening in foals, and although early, specific therapeutic intervention is most successful,[1,2,13,14] some of the most effective and most commonly used therapeutic regimens are associated with potentially fatal adverse effects, which means they should not be used in foals that are not truly infected with *R equi*.[2,15,16] For these reasons, any diagnostic test for *R equi* must be both highly sensitive (ie, unlikely to yield false-negative results) and highly specific (ie, unlikely to yield false-positive results). False-negative results may delay or prevent initiation of specific treatment in a timely manner, and false-positive test results might result in needless treatment of foals that did not have the disease.[17]

A variety of serologic assays that monitor *R equi*-specific antibodies have been developed, primarily for research purposes. These assays have been vitally important in substantiating the existence of a humoral immune response to *R equi* and in characterizing that response in foals.[1,2,7,13] The use of such serologic tests to diagnose *R equi* infection in foals has been proposed, but results of pertinent investigations have, for the most part, been contradictory or equivocal.[18-21] Nevertheless, numerous practicing veterinarians rely on results of serologic assays to establish, confirm, or exclude a diagnosis of *R equi* pneumonia in foals. More recently, serologic testing has been recommended primarily as a surveillance tool to identify foals suspected of being infected, particularly on farms on which the disease is endemic.[2,4]

From the Department of Large Animal Medicine and Surgery, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843 (Martens, Cohen, Chaffin); the Department of Animal Hygiene, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Japan (Takai); Veterinary Dynamics Inc, 1525 Templeton Rd, Templeton, CA 93465 (Doherty); Texas Veterinary Medical Diagnostic Laboratory, 1 Sippel Rd, College Station, TX 77843 (Angulo); and Heart of Texas Equine Clinic, 8533 E Hwy 84, Waco, TX 76705 (Edwards).

Supported by the Link Equine Research Endowment, College of Veterinary Medicine, Texas A&M University, Equine Research Institute, Japan Racing Association, and Veterinary Dynamics Inc.

The authors thank Wade Friedeck, Stephane Robillard, and Dr. Jack Gaskin for technical assistance.

Address correspondence to Dr. Martens.

Three basic types of serologic assays are currently available: ELISA, **agar-gel immunodiffusion (AGID)** assays, and **synergistic hemolysis inhibition (SHI)** assays. Variations within types of assays are based primarily on differences in test antigen preparation and content. However, there is only limited information available regarding sensitivity and specificity of these serologic assays, whether used on single or paired samples. The purpose of the study reported here, therefore, was to determine the sensitivity and specificity of 5 serologic assays used to identify *R equi* infection among foals from a horse-breeding farm on which *R equi* infection was endemic. The assays that were evaluated included 3 ELISA, an AGID assay, and an SHI assay. Results of the assays were analyzed to determine whether a single sample could be used to identify *R equi* infection early in the course of disease (ie, prior to the onset of clinical signs), to determine whether diagnostic accuracy was increased by testing paired serum samples collected during the fourth and sixth or seventh week after birth, and to determine whether *R equi*-infected foals could be differentiated from unaffected foals.

Materials and Methods

The study was conducted on a single, well-managed Arabian horse-breeding farm in Texas; *R equi* infection was considered to be endemic on this farm. For purposes of the present study, a foal was considered to have *R equi* disease if it was between 3 weeks and 6 months old and had clinical signs of pneumonia and if *R equi* had been isolated from a tracheobronchial fluid sample or a lung sample obtained during postmortem examination or the foal had at least 1 of the following signs: multifocal pulmonary opacities on thoracic radiographs or cytologically visible gram-positive intracellular coccobacilli in a tracheobronchial aspirate.

All foals were born on the study farm and remained there until they were at least 6 months old. Serum IgG concentrations were monitored for failure of passive transfer of colostral immunoglobulins, and no foals received transfusions of equine plasma. Foals were observed daily by farm management personnel for signs of disease (eg, malaise, inappetence, unthriftiness, dyspnea, cough, nasal discharge, and diarrhea). The attending veterinarian visited the farm every other weekday and was on call on alternate days to examine any foals suspected of being ill. Physical examination of foals suspected of being ill included thoracic auscultation, thoracic radiography, and a CBC. When deemed important by the attending veterinarian for establishment of a diagnosis, a tracheobronchial aspirate was obtained and submitted for cytologic examination and microbiologic culture. In addition, at 2 to 3 months of age, all foals were screened for respiratory tract disease. The screening procedure consisted of physical examination, thoracic auscultation, thoracic radiography, and a CBC. The incidence of *R equi* pneumonia among foals on the farm during the study period was 43% (17/40).

Blood samples were collected from each foal once during the second, fourth, and sixth or seventh week of life. For foals that developed *R equi* pneumonia, an additional blood sample was collected at the time of diagnosis. For each affected foal from which a blood sample was collected at the time of diagnosis, blood samples were also collected from 2 age-matched (within 1 week of age) unaffected control foals. Blood samples were collected aseptically via jugular venipuncture into evacuated tubes containing a gel barrier and clot activator. Serum was harvested by centrifugation and divided into aliquots, and samples were stored at –70 C until assays were conducted.

After all foals on the farm reached 6 months of age, serum samples from foals that developed *R equi* pneumonia and those that did not develop such disease were segregated. Because a substantial number of age-matched unaffected foals ultimately developed disease, 1 age-matched unaffected foal was used for comparison with each affected foal whenever possible. If samples from 2 age-matched unaffected foals were available, the sample from the foal that was closest in age to the affected foal was selected for comparison. Because some foals were not available for all blood sample collections, the numbers of affected and unaffected foals at each sampling time were not uniform.

Frozen serum samples were coded, so that laboratory personnel were not aware of the disease status of foals from which samples had been collected, and submitted to participating laboratories for analysis. All 5 assays were conducted on aliquots of each serum sample, with the exception that 2 of the ELISA were not performed on samples from 2 affected and 2 unaffected foals.

ELISA—Serum samples were tested with 3 ELISA. Two of these ELISA were performed in the laboratory of 1 of the authors (ST). For these assays, detergent-extracted antigens were prepared from virulent (ATCC No. 33701) and avirulent (ATCC No. 6939) strains of *R equi*, as described.[7,21] Briefly, bacteria were grown on brain heart infusion agar and harvested after 5 days of incubation at 38 C. *Rhodococcus equi* colonies (2 g, wet weight) were suspended in 10 ml of 0.0125*M* sodium phosphate buffer (pH 7.4) containing 0.1% (w/v) Tween 20. Solutions were incubated at 37 C for 90 minutes in a water bath with agitation and centrifuged at 20,000 × g for 30 minutes at 4 C. The supernatant was harvested, adjusted to a concentration of 1.0 µg of protein/ml in carbonate-bicarbonate buffer (pH 9.6), and used as the antigen for the ELISA. Antigens were examined for the 15- to 17-kd VapA by immunoblotting, as described.[22] Antigen preparations from both strains of *R equi* contained surface and capsular antigens, and the antigen preparation from the 33701 strain also contained VapA.

For the ELISA incorporating antigens from strain 6939 (ELISA-6939), 2 cutoff points (**optical density [OD]** of 0.3 and OD of 0.5), above which assay results were considered positive, were used. The cutoff of an OD of 0.3 was selected on the basis of mean OD for healthy foals plus 3 times the SD, as reported.[4,23] The cutoff of an OD of 0.5 was included for comparison with results of the ELISA incorporating antigens from strain 33701 (ELISA-33701). Although mean values for results of the ELISA-33701 in healthy foals have not been determined, the same cutoff points (OD of 0.3 and OD of 0.5) were selected for the purposes of the present study. An OD of 0.5 was selected as a cutoff because the ELISA-33701 is reportedly a more sensitive assay than the ELISA-6939.[7,24]

The third ELISA used in the study consisted of a dilution ELISA, similar to that previously described,[25] and was performed at a commercial laboratory.[a] For this assay, purified recombinant VapA, prepared as described,[26] was used as the antigen. Titers were expressed as the natural logarithm. Briefly, 100 µl of antigen preparation was incubated overnight at 4 C in wells on flat-bottomed polystyrene microtitration plates.[b] Plates were washed and blocked with **0.05% Tween 20 and 0.5% gelatin**[c] **in phosphate-buffered saline solution (PBSS-TG)**, at a pH of 7.2. Serial 2-fold dilutions of test samples were made in PBSS-TG, and 100 µl was added to the wells. Plates were incubated at 37 C for 1 hour and washed in PBSS-TG, and 100 µl of horseradish-peroxidase conjugated goat anti-horse IgG,[d] diluted 1:50,000, was added to each well. Plates were incubated and washed with PBSS-TG, and 100 µl of substrate[e] was added to each well. Plates were incubated a final time for 1 hour at room temperature, and OD of the wells was read with an automated ELISA reader at 405 and 630 nm. Wells with antigen only were used as controls. The ELISA titer was expressed as the last serum dilution giving a reading twice the OD of a negative control serum diluted 1:10. For the ELISA incorporating VapA (ELISA-VapA), the median

value for the highest observed titer for foals in this study was 1,280, so a titer of 640 was selected as the cutoff point above which results of the assay were considered positive.

AGID assay—The AGID assay was performed as described[20,27] at a commercial laboratory.[a] Antigen was obtained from virulent *R equi* strains grown by use of a proprietary method that results in production of large amounts of soluble antigens, including the extracellular enzymes cholesterol oxidase and phospholipase C, and somatic antigens, including virulence-associated proteins (eg, VapA).[27] Bacteria were removed by centrifugation. Positive control samples consisted of serum samples from foals with naturally occurring chronic *R equi* infection that had high concentrations of *R equi*-specific precipitating antibody.

Gel diffusion plates were prepared in 100-mm-diameter Petri dishes with 1% purified agar[f] in borate buffer. Wells (4 mm diameter) were punched in a hexagonal pattern around a central well so that all wells were 3 mm apart, and there were 5 patterns/gel diffusion plate. Antigen (35 µl) was added to each central well, and test samples (45 µl) were added alternately with positive control samples (50 µl) to peripheral wells, so that each hexagonal pattern permitted testing of 3 test samples. Plates were incubated in a humidified chamber at 25 C for 2 days and examined for precipitin lines with indirect lighting.

Paired lots of antigen and positive control serum samples were initially balanced by diluting the antigen so that precipitin lines formed by the positive control serum sample developed midway between the antigen and serum wells and passed straight into adjacent negative test serum wells. Positively reacting test serum samples formed lines of identity with the positive control serum sample and were assigned numerical scores as follows: 0.25 (very weak positive; precipitin lines for the positive control serum sample bend into test serum wells), 0.5 (weak positive; precipitin lines for the positive control serum sample bend in front of test wells), 1.0 (positive; precipitin lines for test serum are comparable to precipitin lines for the positive control serum sample), 2.0 (strong positive; precipitin lines for test serum samples form closer to antigen well than do precipitin lines for the positive control serum sample), and 3.0 to 5.0 (very strong positive; precipitin lines for test serum samples are more diffuse and progressively more intense). On the basis of the assumption that precipitin lines were indicative of a positive test result, sensitivity and specificity of the AGID assay were evaluated at 2 cutoff points (0.25 and 1.0), which corresponded to very weak positive and positive reactions, respectively.

SHI assay—The SHI assay was performed as described[28] at a commercial laboratory.[g] Soluble *R equi* antigens consisting primarily of cholesterol oxidase and phospholipase C were prepared from an *R equi* isolate from a foal with naturally occurring pneumonia. Briefly, *R equi* was grown in tryptose broth for 48 hours at 37 C. The broth was then centrifuged at 1,800 × g for 30 minutes, and the supernatant was filtered through a 22-µm filter.[h] This preparation was tested for synergistic hemolytic activity with β-hemolytic *Staphylococcus aureus* on blood agar plates and stored at –70 C. The highest 2-fold dilution that resulted in complete hemolysis after 48 hours of incubation at 37 C represented 1 unit of *R equi* factor. The 8-fold dilution (8 units) was used in the assay.

A clinical isolate of β-hemolytic *S aureus* was streaked across the diameter of a blood agar plate containing 5% bovine erythrocytes, and the plate was incubated at 37 C for 8 hours. Wells (4 mm diameter) were cut about 8 mm from the *S aureus* streak and filled with 25 µl (8 units) of *R equi* factor or a mixture of *R equi* factor and 2-fold serial dilutions of test serum samples. The plates were incubated for 16 to 24 hours at 37 C or until the *R equi* factor-positive control sample developed a 3- to 4-mm-long semicircular zone of hemolysis. Antibody titers were expressed as the reciprocal of the highest dilution of test serum that inhibited hemolysis, with 1:32 being the highest dilution tested. For calculation of assay sensitivity and specificity, titers ≥ 16 and ≥ 32 were considered indicative of positive assay results.

Statistical analyses—Sensitivity and specificity of each assay were calculated.[17] Continuous and ordinal data (eg, serologic titer) were compared between groups of affected and unaffected foals with the Wilcoxon rank-sum test.[29] Categoric data (eg, proportion of foals that seroconverted) were compared between groups of affected and unaffected foals with χ^2 or Fisher exact tests.[29] Linear mixed-effects models[i] were fit[30] to examine changes in serologic values as a function of time and group (affected vs unaffected). For linear mixed-effect modeling, each individual foal was considered a random effect, and age and group were considered fixed effects. For all analyses, a value of $P < 0.05$ was considered significant.

Results

ELISA-6939—Blood samples collected from 21 foals at 2 weeks of age were tested with the ELISA-6939. Twelve of these foals were classified as affected and 9 were classified as unaffected. Age at which blood samples were collected from affected foals (median, 11 days; range, 7 to 14 days) was not significantly different from age at which blood samples were collected from unaffected foals (8 days; range, 7 to 13 days). Median OD for the affected foals (0.150; range, 0.092 to 0.225) was significantly ($P = 0.013$) lower than median OD for the unaffected foals (0.237; range, 0.123 to 0.388). However, although specificity of the assay was moderate or high, depending on the cutoff used, sensitivity was 0 (**Table 1**).

Table 1—Sensitivity and specificity of an ELISA incorporating antigen from an avirulent strain of *Rhodococcus equi* (ATCC No. 6939) performed on sequential serum samples from foals

Sample collection time and foal status	OD > 0.3 considered a positive result				OD > 0.5 considered a positive result			
	No. of foals		Sensitivity (%)	Specificity (%)	No. of foals		Sensitivity (%)	Specificity (%)
	Positive	Negative			Positive	Negative		
2 weeks of age			0	67			0	100
Affected	0	12			0	12		
Unaffected	3	6			0	9		
4 weeks of age			0	89			0	100
Affected	0	11			0	11		
Unaffected	1	8			0	9		
6 to 7 weeks of age			40	70			10	90
Affected	4	6			1	9		
Unaffected	3	7			1	9		
Time of clinical diagnosis			58	50			42	100
Affected	7	5			5	7		
Unaffected	4	4			0	8		

OD = Optical density.
A foal was considered to be affected if it had clinical signs of pneumonia when it was between 3 weeks and 6 months old and *R equi* was isolated from a tracheobronchial fluid sample or lung sample obtained during postmortem examination or multifocal pulmonary opacities were present on thoracic radiographs or gram-positive intracellular coccobacilli were seen in a tracheobronchial aspirate.

Blood samples collected from 20 foals (11 affected and 9 unaffected) at 4 weeks of age were tested with the ELISA-6939. Age at which blood samples were collected from affected foals (median, 25 days; range, 21 to 27 days) was not significantly different from age at which blood samples were collected from unaffected foals (23 days; range, 21 to 26 days). Median OD for the affected foals (0.159; range, 0.107 to 0.233) was not significantly different from median OD for the unaffected foals (0.155; range, 0.113 to 0.305). Although specificity was high, sensitivity was 0 (Table 1).

Table 2—Sensitivity and specificity of an ELISA incorporating antigen from a virulent strain of *R equi* (ATCC No. 33701) performed on sequential serum samples from foals

Sample collection time and foal status	OD > 0.3 considered a positive result: No. of foals Positive	Negative	Sensitivity (%)	Specificity (%)	OD > 0.5 considered a positive result: No. of foals Positive	Negative	Sensitivity (%)	Specificity (%)
2 weeks of age			0	78			0	100
Affected	0	12			0	12		
Unaffected	2	7			0	9		
4 weeks of age			0	89			0	100
Affected	0	11			0	11		
Unaffected	1	8			0	9		
6 to 7 weeks of age			40	40			10	80
Affected	4	6			1	9		
Unaffected	6	4			2	8		
Time of clinical diagnosis			50	38			50	88
Affected	6	6			6	6		
Unaffected	5	3			1	7		

See Table 1 for key.

Blood samples collected from 20 foals (10 affected and 10 unaffected) at 6 to 7 weeks of age were tested with the ELISA-6939. Age at which blood samples were collected from affected foals (median, 45.5 days; range, 40 to 51 days) was not significantly different from age at which blood samples were collected from unaffected foals (43.5 days; range, 36 to 54 days). Median OD for the affected foals (0.215; range, 0.107 to 0.541) was not significantly different from median OD for the unaffected foals (0.226; range, 0.126 to 1.210). Specificity was moderate or high, but sensitivity was low (Table 1).

Blood samples were collected from 12 affected foals at the time of diagnosis and tested with the ELISA-6939; results for 8 age-matched unaffected foals were available for comparison. Age at the time of diagnosis for the affected foals (median, 70 days; range, 34 to 148 days) was not significantly different from age at the time of blood sample collection for unaffected foals (71.5 days; range, 61 to 131 days). Median OD for the affected foals (0.400; range, 0.152 to 1.952) was not significantly different from median OD for the unaffected foals (0.290; range 0.181 to 0.449). Sensitivity was moderate and specificity was low at the lower cutoff but 100% at the higher cutoff (Table 1).

ELISA-33701—The same blood samples tested with the ELISA-6939 were tested with the ELISA-33701. For samples collected from foals at 2 weeks of age, median OD for the affected foals (0.152; range, 0.016 to 0.283) was not significantly different from median OD for the unaffected foals (0.226; range 0.093 to 0.484). Specificity was high, but sensitivity was 0 **(Table 2)**.

For samples collected from the foals at 4 weeks of age, median OD for the affected foals (0.161; range, 0.105 to 0.242) was not significantly different from median OD for the unaffected foals (0.159; range, 0.109 to 0.337). Specificity was high, but sensitivity was still 0 (Table 2).

For samples collected from the foals at 6 to 7 weeks of age, median OD for the affected foals (0.232; range, 0.149 to 0.678) was not significantly different from median OD for the unaffected foals (0.328; range, 0.145 to 0.560). Sensitivity was low and specificity was low or moderate (Table 2).

For samples collected from 12 foals at the time of diagnosis and from 8 age-matched unaffected foals, median OD for the affected foals (0.396; range, 0.147 to 1.686) was not significantly different from median OD for the unaffected foals (0.331; range, 0.191 to 0.712). Sensitivity was low and specificity was low or moderate (Table 2).

ELISA-VapA—Blood samples collected from 25 foals (14 affected and 11 unaffected) at 2 weeks of age were tested with the ELISA-VapA. Age at which blood samples were collected from affected foals (median, 11 days; range, 7 to 14 days) was not significantly different from age at which blood samples were collected from unaffected foals (9 days; range, 7 to 13 days). Median titer for the affected foals (160; range, 20 to 1,280) was not significantly different from median titer for the unaffected foals (160; range, 40 to 180). Sensitivity was low and specificity was high **(Table 3)**.

Blood samples collected from 24 foals (13 affected

Table 3—Sensitivity and specificity of an ELISA incorporating virulence-associated protein antigen performed on sequential serum samples from foals

Sample collection time and foal status	Titer > 640 considered a positive result: No. of foals Positive	Negative	Sensitivity (%)	Specificity (%)
2 weeks of age			7	91
Affected	1	13		
Unaffected	1	10		
4 weeks of age			8	100
Affected	1	12		
Unaffected	0	11		
6 to 7 weeks of age			29	75
Affected	4	10		
Unaffected	3	9		
Time of clinical diagnosis			42	50
Affected	5	7		
Unaffected	4	4		

See Table 1 for key.

and 11 unaffected) at 4 weeks of age were tested with the ELISA-VapA. Age at which blood samples were collected from affected foals (median, 25 days; range, 21 to 27 days) was not significantly different from age at which blood samples were collected from unaffected foals (24 days; range, 21 to 26 days). Median titer for the affected foals (160; range, 0 to 5,120) was not significantly different from median titer for the unaffected foals (40; range, 20 to 640). Sensitivity was low and specificity was high (Table 3).

Blood samples collected from 26 foals (14 affected and 12 unaffected) at 6 to 7 weeks of age were tested with the ELISA-VapA. Age at which blood samples were collected from affected foals (median, 46.5 days; range, 40 to 51 days) was not significantly different from age at which blood samples were collected from unaffected foals (44.5 days; range, 36 to 54 days). Median titer for the affected foals (320; range, 20 to 5,120) was not significantly different from median titer for the unaffected foals (240; range, 10 to 10,240). Sensitivity was low and specificity was moderate (Table 3).

Blood samples collected from the 12 affected foals at the time of diagnosis and from the 8 age-matched unaffected foals tested with the ELISA-6939 were also tested with the ELISA-VapA. Median titer for the affected foals (640; range, 40 to 10,240) was not significantly different from median titer for the unaffected foals (960; range, 160 to 1,280). Sensitivity and specificity were both low (Table 3).

AGID assay—Blood samples collected from 25 foals (14 affected and 11 unaffected) at 2 weeks of age were tested with the AGID assay. Age at which blood samples were collected from affected foals (median, 11 days; range, 7 to 14 days) was not significantly different from age at which blood samples were collected from unaffected foals (9 days; range, 7 to 13 days). Median test value for the affected foals (0.5; range, 0.25 to 1.0) was not significantly different from median test value for the unaffected foals (0.5; range, 0.25 to 1.0). Sensitivity was 100% and specificity was 0 at a cutoff of 0.25; sensitivity was 0 and specificity was 100% at a cutoff of 1.0 (**Table 4**).

Blood samples collected from 24 foals (13 affected and 11 unaffected) at 4 weeks of age were tested with the AGID assay. Age at which blood samples were collected from affected foals (median, 25 days; range, 21 to 27 days) was not significantly different from age at which blood samples were collected from unaffected foals (24 days; range, 21 to 26 days). Median test value for the affected foals (0.5; range, 0.25 to 2.0) was not significantly different from median test value for the unaffected foals (0.5; range, 0.25 to 2.0). Sensitivity was 100% and specificity was 0 at a cutoff of 0.25; sensitivity was low and specificity was high at a cutoff of 1.0 (Table 4).

Blood samples collected from 26 foals (14 affected and 12 unaffected) at 6 to 7 weeks of age were tested with the AGID assay. Age at which blood samples were collected from affected foals (median, 46.5 days; range, 40 to 51 days) was not significantly different from age at which blood samples were collected from unaffected foals (44.5 days; range, 36 to 54 days). Median test value for the affected foals (1.5; range, 0.5 to 5) was not significantly different from median test value for the unaffected foals (2; range, 0.5 to 5). Sensitivity was 100% and specificity was 0 at a cutoff of 0.25; both sensitivity and specificity were low at a cutoff of 1.0 (Table 4).

Blood samples collected from 13 affected foals at the time of diagnosis and from 8 age-matched unaffected foals were also tested with the AGID assay. Age at which blood samples were collected from affected foals (72 days; range, 34 to 148 days) was not significantly different from age at which blood samples were collected from unaffected foals (71.5 days; range, 61 to 131 days). Median test value for the affected foals (3; range, 1.0 to 5.0) was not significantly different from median test value for the unaffected foals (2; range, 1.0 to 5.0). Sensitivity was high and specificity was low (Table 4).

SHI assay—The same blood samples tested with the AGID assay were tested with the SHI assay. For samples collected from all foals at 2 weeks of age, titer was 0. Sensitivity was 0 and specificity was 100% (**Table 5**). Similarly, for samples collected from all foals at 4 weeks of age, titer was 0; sensitivity was 0 and specificity was 100%.

For samples collected from the foals at 6 to 7 weeks of age, median titer for the affected foals (0; range, 0 to ≥ 32) was not significantly different from median titer for the unaffected foals (0; range, 0 to ≥ 32). Sensitivity was low and specificity was high (Table 5).

For samples collected from 13 affected foals at the time of diagnosis and from 8 age-matched unaffected foals, median titer for the affected foals (16; range, 0 to ≥ 32) was not significantly different from median titer for the unaffected foals (8; range, 0 to ≥ 32). Sensitivity and specificity were both low or moderate (Table 5).

Table 4—Sensitivity and specificity of an agar gel immunodiffusion assay performed on sequential serum samples from foals

Sample collection time and foal status	Score ≥ 0.25 considered a positive result				Score ≥ 1.0 considered a positive result			
	No. of foals		Sensitivity (%)	Specificity (%)	No. of foals		Sensitivity (%)	Specificity (%)
	Positive	Negative			Positive	Negative		
2 weeks of age			100	0			0	100
Affected	14	0			0	14		
Unaffected	11	0			0	11		
4 weeks of age			100	0			8	91
Affected	13	0			1	12		
Unaffected	11	0			1	10		
6 to 7 weeks of age			100	0			50	42
Affected	14	0			7	7		
Unaffected	12	0			7	5		
Time of clinical diagnosis			100	0			85	13
Affected	13	0			11	2		
Unaffected	8	0			7	1		

See Table 1 for key.

Table 5—Sensitivity and specificity of a synergistic hemolysis inhibition assay performed on sequential serum samples from foals

Sample collection time and foal status	Titer ≥ 16 considered a positive result				Titer ≥ 32 considered a positive result			
	No. of foals		Sensitivity (%)	Specificity (%)	No. of foals		Sensitivity (%)	Specificity (%)
	Positive	Negative			Positive	Negative		
2 weeks of age			0	100			0	100
Affected	0	14			0	14		
Unaffected	0	11			0	11		
4 weeks of age			0	100			0	100
Affected	0	13			0	13		
Unaffected	0	11			0	11		
6 to 7 weeks of age			14	83			7	92
Affected	2	12			1	13		
Unaffected	2	10			1	11		
Time of clinical diagnosis			54	50			46	63
Affected	7	6			6	7		
Unaffected	4	4			3	5		

See Table 1 for key.

Evaluation of paired serum samples—For the ELISA-6939, results for paired samples collected at 4 weeks of age and 6 to 7 weeks of age were available for 9 affected and 9 unaffected foals. The proportion (8/9) of affected foals with an increase in assay results was not significantly different from the proportion (5/9) of unaffected foals with an increase. Sensitivity of using any increase in the ELISA-6939 OD as indicative of *R equi* infection was 89% (8/9), but specificity was only 44% (4/9).

Foals were considered to have seroconverted if OD at 4 weeks of age was < 0.3 and OD at 6 to 7 weeks of age was > 0.3. The proportion (3/9) of affected foals that seroconverted was not significantly different than the proportion (2/9) of unaffected foals that seroconverted. Sensitivity of using seroconversion as being indicative of *R equi* infection was 33% (3/9), and specificity was 79% (7/9). Only 1 foal had an OD < 0.5 at 4 weeks of age and > 0.5 at 6 to 7 weeks of age; this was an affected foal.

Results of the ELISA-33701 were also available for paired samples collected at 4 weeks of age and 6 to 7 weeks of age from 9 affected and 9 unaffected foals. Proportions (8/9) of foals that had an increase in OD were identical for affected and unaffected foals. Sensitivity of using any increase in the ELISA-33701 OD as indicative of *R equi* infection was 89% (8/9), but specificity was only 11% (1/9).

Foals were considered to have seroconverted if OD at 4 weeks of age was < 0.3 and OD at 6 to 7 weeks of age was > 0.3. The proportion (3/9) of affected foals that seroconverted was not significantly different than the proportion (5/9) of unaffected foals that seroconverted. Sensitivity of using seroconversion as being indicative of *R equi* infection was 33% (3/9), and specificity was 44% (4/9). Three foals had an OD < 0.5 at 4 weeks of age and > 0.5 at 6 to 7 weeks of age; 1 was affected and 2 were unaffected.

For the ELISA-VapA, results for paired samples collected at 4 weeks of age and 6 to 7 weeks of age were available for 13 affected and 11 unaffected foals. The proportion (7/13) of affected foals with an increase in assay results was not significantly different from the proportion (7/11) of unaffected foals with an increase. Sensitivity of using any increase in the ELISA-VapA titer as indicative of *R equi* infection was 54% (7/13), but specificity was only 36% (4/11). The proportion (2/13) of affected foals that seroconverted (eg, titer < 640 at 4 weeks of age and > 640 at 6 to 7 weeks of age) was not significantly different from the proportion (3/11) of unaffected foals that seroconverted. Three affected and 6 unaffected foals had a 4-fold increase in titer between 4 weeks of age and 6 to 7 weeks of age.

For the AGID assay, results for paired samples collected at 4 weeks of age and 6 to 7 weeks of age were available for 13 affected and 11 unaffected foals. The proportion (9/13) of affected foals with an increase in assay results was not significantly different from the proportion (8/11) of unaffected foals with an increase. Sensitivity of using any increase in the AGID assay result as indicative of *R equi* infection was 69% (9/13), but specificity was only 27% (3/11).

For the SHI assay, results for paired samples collected at 4 weeks of age and 6 to 7 weeks of age were available for 13 affected and 11 unaffected foals. The proportion (2/13) of affected foals that had an increase in SHI titer was not significantly different from the proportion (2/11) of unaffected foals with an increase. Sensitivity of using any increase in SHI titer as indicative of *R equi* infection was 15% (2/13), and specificity was 82% (9/11). Two foals had a titer of 0 at 4 weeks of age and a titer ≥ 32 at 6 to 7 weeks of age; 1 was affected and 1 was unaffected.

Changes in titer over time—Linear mixed-effect modeling indicated that for all 5 assays, there was a significant increase in assay values over time. However, there were no significant differences between affected and unaffected foals in regard to the rate of increase.

Discussion

Results of the present study suggested that none of the 5 serologic assays could be used to differentiate between *R equi*-affected foals and unaffected foals at any of the testing times. In addition, diagnostic accuracy was not increased by testing paired serum samples collected at 4 weeks of age and at 6 to 7 weeks of age, in that for all 5 assays, proportions of affected and unaffected foals that had increases in assay values were not significantly different. Finally, evaluation of sensitivity and specificity of the 5 assays indicated that none of them could be used to differentiate *R equi*-affected foals from unaffected foals at the time of clinical diagnosis. It appears, therefore, that these serologic assays do not reliably detect early stages of disease or identify

affected foals by the time a specific diagnosis can be accomplished with standard diagnostic methods.

Results of the serologic assays used in the present study did suggest that in the broadest sense, all foals in the present study were infected with *R equi*, in that assay results increased in all foals over time and development of antibodies to a microorganism necessitates colonization of host cells by that organism.[31] This agrees with results of previous studies[1,2,5,7] in which a high prevalence of *R equi* antibodies in the horse population suggested that virtually all horses are or have been infected with *R equi*. Fortunately, however, only a small proportion of these infections result in overt clinical disease. Overt clinical disease in the form of pneumonia was the outcome of interest in the present study.

Serologic assays that incorporate antigens common to all virulent and avirulent strains of *R equi* (ie, the ELISA-6939, ELISA-33701, AGID assay, and SHI assay in the present study) assess exposure to virulent and avirulent strains of the organism, whereas serologic assays that incorporate only virulence-associated antigens (ie, the ELISA-VapA) would be expected to monitor exposure only to virulent strains of *R equi*. A recent study,[6] however, found that there was no direct association between the presence of virulent or avirulent *R equi* in the soil of horse-breeding farms and the presence or absence of clinical *R equi* disease on those farms. Consequently, many foals may be exposed to virulent and avirulent strains of *R equi* and develop corresponding serum antibodies.[24] Foals residing at farms on which *R equi* infection is endemic are more likely to be exposed to high concentrations of the bacterium[7,13]; however, the extent of exposure and immune responsiveness of exposed foals may vary.

Serologic testing has apparently been quite useful in the management of *R equi* infection in some practice situations.[27,32] In general, however, there is a decreased emphasis on its use as a diagnostic tool because of the widespread exposure of foals to the organism and the low percentage of exposed foals that develop clinical disease. In addition, exposure to virulent or avirulent strains may result in measurable serum antibodies, and maternally derived antibodies may confound test results. Beyond this, there is no evidence that the presence or magnitude of a humoral response is associated with whether clinical disease will develop, and some foals that do not develop clinical disease seroconvert, whereas other foals that do develop clinical disease do not.[2,33]

Several recent studies[4,7,23,24] have investigated the diagnostic usefulness of the ELISA-6939 and ELISA-33701. In those studies, the ELISA-6939 was determined to be more suitable for the diagnosis of disease than the ELISA-33701, because the latter assay was less specific. Because sensitivity and specificity of the ELISA-6939 had not been established, however, it was recommended that a diagnosis of *R equi* pneumonia not be made solely on the basis of seroconversion.[4] Results of the present study indicated that sensitivities and specificities of the 2 assays were similar and that neither assay was suitable for diagnosis of *R equi* pneumonia. The finding that ELISA-6939 values were significantly greater for unaffected foals than for affected foals at the second week of age is difficult to explain and most likely represents a random occurrence. This difference did not extend to other age groups.

Sensitivity and specificity should be taken into consideration when a clinical test is selected. A highly sensitive test should be used when it is important not to miss a diagnosis (eg, when an animal is suspected to have a dangerous but treatable disease). Highly specific tests are most useful to confirm a tentative diagnosis, because they rarely provide positive results in the absence of disease. Highly specific tests are particularly important if false-positive results could harm patients. The ideal test is 1 that is both highly sensitive (few false-negative results) and highly specific (few false-positive results); however, such tests are rare. In particular, for tests with continuous results, a cutoff is selected that is a trade-off between sensitivity and specificity. In these instances, sensitivity generally can only be increased at the expense of specificity, and vice versa.[17] *Rhodococcus equi* pneumonia is a dangerous disease that responds well if specific treatment is initiated early in the course of infection. There are, however, risks associated with some of the most successful therapeutic regimens.[2,15,16] On the basis of the probability of success with these regimens,[34] imposition of these risks is usually warranted in foals with *R equi* pneumonia, whereas these risks may not be justified in foals that do not have the disease. Thus, high specificity is an important property of a test for *R equi* pneumonia in foals.

In most instances in the present study, assays had high sensitivity and negligible specificity or negligible sensitivity and high specificity. There were only 3 instances wherein sensitivity and specificity both equaled or exceeded 50%, and all 3 of these instances involved testing of samples after the disease had already been diagnosed by standard diagnostic methods. The low degree of diagnostic accuracy represented by these values does not provide a sufficient basis on which to establish a diagnosis and thereby determine whether to initiate or withhold specific treatment for *R equi* pneumonia.

Predictive values are another measure of the reliability of diagnostic tests that are commonly used by clinicians; however, they were not calculated in the present study because of the low sensitivities and specificities that were obtained. In addition, the high prevalence of disease among foals in this study would have artifactually enhanced positive predictive values and diminished negative predictive values.

Diagnosis of infectious diseases, with the exception of persistent infections such as equine infectious anemia, is usually not based on serologic assessment of a single sample. Serologic testing is most appropriately used to confirm the presence or absence of a suspected disease, and such confirmation is most commonly based on detecting an increase in titer of specific serum antibodies (seroconversion) between a serum sample collected at the initiation of clinical signs and a sample collected approximately 2 to 3 weeks later. With a chronic disease such as *R equi* pneumonia, however, such a marked increase in antibody titer would not be expected, because patients have been infected for a

prolonged period by the time clinical signs become apparent. Antibody titer may have already peaked by the time clinical signs of disease are apparent and, in fact, may be declining because of consumption. In the present study, none of the assays had both a sensitivity ≥ 50% and a specificity ≥ 50% when any increase in test value or seroconversion between the fourth and sixth or seventh week of age was used as an indication of *R equi* disease. These time periods were selected on the basis of a previous study[23] involving the ELISA-6939.

Results of this investigation might have been modified somewhat if foals were included from farms on which *R equi* pneumonia was sporadic or nonexistent. Because of reduced exposure to *R equi* among foals on such farms, it is expected that results of serologic tests would have been similar to those in a recent study[23] in which seroconversion rates were greatest among foals on farms on which *R equi* pneumonia was endemic, moderate on farms with sporadic infections, and lowest on farms with no history of *R equi* infection. However, although positive and negative predictive values may have changed somewhat with a different population of foals, the sensitivities and specificities of the assays should not have been substantially different. Because veterinary practitioners would most likely consider using these serologic tests on foals from farms on which *R equi* infection was endemic,[2,4,20] it was deemed most appropriate to evaluate the assays in a similar environment.

Statistical power was limited in this study by the small sample size. Nevertheless, the study had sufficient statistical power to detect differences in assay results of approximately 50% between groups. For comparisons of proportions of foals that had increased titers or seroconverted, statistical power was limited; however, the magnitude of differences between groups was generally small, and these differences did not appear clinically important. Overall, limited statistical power did not seem to explain the poor correlation between serologic test results and clinical status of the study foals.

Historically, serologic testing has been an important component of the diagnostic armamentarium available to veterinarians. However, serologic testing is most effectively used to substantiate a provisional diagnosis of acute or subacute infection, and because paired serum samples are most often used, results are oftentimes retrospective in nature. *Rhodococcus equi* infection presents a diagnostic challenge, particularly during the early stages of disease, prior to the onset of clinical signs, when treatment should be most effective. The hope that serologic tests could be effectively used to identify infected foals is appealing, and 3 of the serologic assays investigated in this study (ie, the ELISA-VapA, AGID assay, and SHI assay) are commercially available in the United States. Numerous veterinarians have relied on these assays to establish, confirm, or exclude a diagnosis of *R equi* pneumonia. On the basis of results of the present study, however, the authors do not believe that serologic assays, whether used to evaluate a single blood sample or used to evaluate paired samples for evidence of seroconversion, are reliable for the diagnosis of *R equi* pneumonia. In addition, because of the high probability of false-positive and false-negative results, it appears as though serologic assessment is not an effective screening tool for identification of foals suspected of having *R equi* pneumonia. Clinical screening procedures such as careful visual inspection, daily monitoring of rectal temperature, thoracic auscultation, CBC, thoracic radiography, and thoracic ultrasonography are probably more useful in identifying foals likely to be in the early stages of disease.[33] If deemed appropriate on the basis of results of these screening procedures, additional diagnostic tests such as microbiologic culture, polymerase chain reaction assays, and cytologic analysis of transtracheal aspirates and blood could be performed to establish a more definitive diagnosis.[2,33,35]

[a]Veterinary Dynamics, Templeton, Calif.
[b]Immulon 1, 96-well, Dynatech Laboratories, Chantilly, Va.
[c]HiPure Liquid Gelatin, Norland Products Inc, New Brunswick, NJ.
[d]Peroxidase-conjugated AffiniPure Goat Anti-horse IgG (H+L), Jackson ImmunoResearch Laboratories Inc, West Grove, Pa.
[e]ABTS, Boehringer Mannheim, St Joseph, Mo.
[f]Difco Agar Noble, Becton Dickinson, Sparks, Md.
[g]Texas Veterinary Medical Diagnostic Laboratory, College Station, Tex.
[h]Millipore Corp, Bedford, Mass.
[i]S-PLUS 2000, Mathsoft Inc, Seattle, Wash.

References

1. Prescott JF. *Rhodococcus equi*: an animal and human pathogen. *Clin Microbiol Rev* 1991;4:20–34.
2. Giguere S, Prescott JF. Clinical manifestations, diagnosis, treatment, and prevention of *Rhodococcus equi* infections in foals. *Vet Microbiol* 1997;56:313–334.
3. Horowitz ML, Cohen ND, Takai S, et al. Application of Sartwell's model (logarithmic-normal distribution of incubation periods) to age at onset and age at death of foals with *Rhodococus equi* pneumonia as evidence of perinatal infection. *J Vet Intern Med* 2001; 15:171–175.
4. Higuchi T, Hashikura S, Gojo C, et al. Clinical evaluation of the serodiagnostic value of enzyme-linked immunosorbent assay for *Rhodococcus equi* infection in foals. *Equine Vet J* 1997;29:274–278.
5. Takai S. Epidemiology of *Rhodococcus equi* infections: a review. *Vet Microbiol* 1997;56:167–176.
6. Martens RJ, Takai S, Cohen ND, et al. Association of disease with isolation and virulence of *Rhodococcus equi* from farm soil and foals with pneumonia. *J Am Vet Med Assoc* 2000;217:220–225.
7. Takai S, Hidaka D, Masunobu F, et al. Serum antibody responses of foals to virulence-associated 15- to 17-kilodalton antigens of *Rhodococcus equi*. *Vet Microbiol* 1996;52:63–71.
8. Martens RJ, Martens JG, Fiske RA, et al. *Rhodococcus equi* foal pneumonia: protective effects of immune plasma in experimentally infected foals. *Equine Vet J* 1989;21:249–255.
9. Madigan JE, Hietala S, Muller N. Protection against naturally acquired *Rhodococcus equi* pneumonia in foals by administration of hyperimmune plasma. *J Reprod Fertil* 1991;44:571–578.
10. Higuchi T, Arakawa T, Hashikura S, et al. Effect of prophylactic administration of hyperimmune plasma to prevent *Rhodococcus equi* infection of foals from endemically affected farms. *Zentralbl Veterinarmed [B]* 1999;46:641–648.
11. Hurley JR, Begg AP. Failure of hyperimmune plasma to prevent pneumonia caused by *Rhodococcus equi* in foals. *Aust Vet J* 1995;72:418–420.
12. Giguere S, Gaskin JM, Miller C, et al. Evaluation of a commercially available hyperimmune plasma product for prevention of naturally acquired pneumonia caused by *Rhodococcus equi* in foals. *J Am Vet Med Assoc* 2002;220:59–63.
13. Takai S, Sasaki Y, Tsubaki S. *Rhodococcus equi* infection in foals: current concepts and implications for future research. *J Equine Sci* 1995;6:105–119.
14. Jacks S, Giguere S, Gronwall RR, et al. Pharmacokinetics of

azithromycin and concentration in body fluids and bronchoalveolar cells in foals. *Am J Vet Res* 2001;62:1870–1875.

15. Stratton-Phelps M, Wilson WD, Gardner IA. Risk of adverse effects in pneumonic foals treated with erythromycin versus other antibiotics: 143 cases (1986–1996). *J Am Vet Med Assoc* 2000;217: 68–73.

16. Baverud V, Franklin A, Gunnarsson A. *Clostridium difficile* associated with acute colitis in mares when their foals are treated with erythromycin and rifampicin for *Rhodococcus equi* pneumonia. *Equine Vet J* 1998;30:482–488.

17. Fletcher RH, Fletcher SW, Wagner EH. *Clinical epidemiology—the essentials*. 2nd ed. Baltimore: The Williams & Wilkins Co, 1988;42–75.

18. Prescott JF, Coshan-Gauthier R, Barksdale L. Antibody to equi-factor(s) in the diagnosis of *Corynebacterium equi* pneumonia of foals. *Can J Comp Med* 1984;48:370–373.

19. Nakazawa M, Isayama Y, Kashiwazaki M. Diagnosis of *Rhodococcus equi* infection in foals by agar gel diffusion test with protein antigen. *Vet Microbiol* 1987;15:105–113.

20. Gaskin JM, King RR, Lane TJ, et al. Serological detection of *Rhodococcus equi* infections in foals, in *Proceedings*. Annu Vet Med Forum 1990;8:581–584.

21. Takai S, Kawazu S, Tsubaki S. Enzyme-linked immunosorbent assay for diagnosis of *Corynebacterium (Rhodococcus) equi* infection in foals. *Am J Vet Res* 1985;46:2166–2170.

22. Takai S, Iie M, Kobayashi C, et al. Monoclonal antibody specific to virulence-associated 15- to 17-kilodalton antigens of *Rhodococcus equi*. *J Clin Microbiol* 1993;31:2780–2782.

23. Higuchi T, Taharaguchi S, Hashikura, et al. Physical and serologic examinations of foals at 30 and 45 days of age for early diagnosis of *Rhodococcus equi* infection on endemically infected farms. *J Am Vet Med Assoc* 1998;212:976–981.

24. Takai S, Hisashi K, Yoshihiro T, et al. Humoral antibody response to the antigens of virulent *Rhodococcus equi* in foals. *J Equine Sci* 1994;5:121–126.

25. Prescott JF, Fernandez AS, Nicholson MC, et al. Use of a virulence-associated protein based enzyme-linked immunosorbent assay for *Rhodococcus equi* serology in horses. *Equine Vet J* 1996; 28:344–349.

26. Hooper-McGrevy KE, Giguere S, Wilkie BN, et al. Evaluation of equine immunoglobulin specific for *Rhodococcus equi* virulence-associated proteins A and C for use in protecting foals against *Rhodococcus equi*-induced pneumonia. *Am J Vet Res* 2001;62: 1307–1313.

27. Becu T, Polledo G, Gaskin JM. Immunoprophylaxis of *Rhodococcus equi* pneumonia in foals. *Vet Microbiol* 1997;56: 193–204.

28. Eugster AK, Whitford HW, Angulo AB. A study of serological tests to measure antibodies to *Rhodococcus (Corynebacterium) equi* factors, in *Proceedings*. 29th Annu Meet Am Assoc Vet Lab Diagn 1986;357–366.

29. Rosner B. In: *Fundamentals of biostatistics*. 2nd ed. Boston: Duxbury Press, 1986;288–293, 302–346.

30. Pinheiro JC, Bates DM. *Mixed effects models in S and S-PLUS*. New York: Springer-Verlag, 2000;1–270.

31. Prescott LM, Harley JP, Klein DA. *Microbiology*. 4th ed. Boston: McGraw-Hill Book Co, 1999;580–604.

32. Wilkins PA, Lesser FR, Gaskin JM. *Rhodococcus equi* pneumonia in foals: comparison of ELISA and AGID serology on a commercial Thoroughbred breeding farm, in *Proceedings*. 39th Annu Conv Am Assoc Equine Pract 1993;289.

33. Cohen ND, Chaffin MK, Martens RJ. Control and prevention of *Rhodococcus equi* pneumonia in foals. *Compend Contin Educ Pract Vet* 2000;22:1062–1070.

34. Hillidge CJ. Use of erythromycin-rifampin combination in treatment of *Rhodococcus equi* pneumonia. *Vet Microbiol* 1987;14: 337–342.

35. Sellon DC, Walker K, Suyemoto M, et al. Nucleic acid amplification for rapid detection of *Rhodococcus equi* in equine blood and tracheal wash fluids. *Am J Vet Res* 1997;58:1232–1237.