Evaluation of fecal samples from mares as a source of *Rhodococcus equi* for their foals by use of quantitative bacteriologic culture and colony immunoblot analyses

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**Objective**—To determine whether mares are a clinically important source of *Rhodococcus equi* for their foals.

**Sample Population**—171 mares and 171 foals from a farm in Kentucky (evaluated during 2004 and 2005).

**Procedures**—At 4 time points (2 before and 2 after parturition), the total concentration of *R. equi* and concentration of virulent *R. equi* were determined in fecal specimens from mares by use of quantitative bacteriologic culture and a colony immunoblot technique, respectively. These concentrations for mares of foals that developed *R. equi*-associated pneumonia and for mares with unaffected foals were compared. Data for each year were analyzed separately.

**Results**—*R. equi*-associated pneumonia developed in 53 of 171 (31%) foals. Fecal shedding of virulent *R. equi* was detected in at least 1 time point for every mare; bacteriologic culture results were positive for 62 of 171 (36%) mares at all time points. However, compared with dams of unaffected foals, fecal concentrations of total or virulent *R. equi* in dams of foals with *R. equi*-associated pneumonia were not significantly different.

**Conclusions and Clinical Relevance**—Results indicate that dams of foals with *R. equi*-associated pneumonia did not shed more *R. equi* in feces than dams of unaffected foals; therefore, *R. equi* infection in foals was not associated with comparatively greater fecal shedding by their dams. However, detection of virulent *R. equi* in the feces of all mares during at least 1 time point suggests that mares can be an important source of *R. equi* for the surrounding environment. (Am J Vet Res 2007;68:63–71)

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*Rhodococcus equi* is a facultative intracellular organism and an important cause of disease and death among foals, particularly on farms where the organism is endemic.1,2 *Rhodococcus equi* is primarily a soil saprophyte with growth requirements that are met by herbivore manure and warm climates.2 *Rhodococcus equi* can be cultured from the feces of mature horses3 and foals as young as 3 days of age.4 It has been suggested that *R. equi* is likely part of the normal microflora in the lower portions of the gastrointestinal tract in horses.3 Among foals, pneumonia caused by *R. equi* is endemic on some farms and develops intermittently on others, but is absent on most farms.2,5 Anecdotally, some mares have had multiple affected foals, whereas foals of other mares from the same environment are consistently unaffected.6 Epidemiologic evidence indicates that foals with naturally occurring *R. equi*-associated pneumonia become infected early in life.7 The source of infection for foals remains unknown. Results of previous studies8-10 indicate that the feces of mares are a potential source of *R. equi* for the environment and possibly a direct source of infection for foals. *Rhodococcus equi* has been isolated from the feces of mares at 2 breeding farms in the United States.6 In a report6 from Australia, the prevalence of fecal shedding of *R. equi* among...
127 horses was 71%. Evidence from Japan has revealed a seasonal variation in shedding of \( R_{equi} \) in feces of mares; the highest levels of \( R_{equi} \) shedding were detected during spring (foaling season), although the mean number of organisms shed by mares during the 5-week periods before and after foaling was not significantly different.10

Expression of VapA by \( R_{equi} \) is strongly associated with disease in foals; the \( \text{vapA} \) gene is located on an 85- to 90-kilobase plasmid.4,5,11 Because only virulent organisms are considered to cause disease, it is important to determine the virulence status of isolates in epidemiologic studies of \( R_{equi} \). In a study12 of 2 breeding farms in Japan, the prevalence of virulent \( R_{equi} \) organisms in feces was greater among foals at an \( R_{equi} \)-endemic farm than at an unaffected farm, although differences in the prevalence of virulent isolates in the feces of their dams were not detected.

To the authors’ knowledge, no studies have been reported in which the concentration of virulent \( R_{equi} \) in fecal samples of mares or foals was determined and the association of these concentrations with disease in foals caused by this bacterium was examined.

Given the high prevalence of \( R_{equi} \) in feces of breeding mares, the large volume of feces passed by adult horses, and the reported increased prevalence of fecal shedding of \( R_{equi} \) by mares during the foaling season, it is plausible that a mare could be a source of virulent \( R_{equi} \) for its foal. To determine whether mares are a clinically important source of \( R_{equi} \) for their foals, the study reported here was undertaken to examine the association between \( R_{equi} \)-associated pneumonia and foals that were born at the farm that developed \( R_{equi} \)-associated pneumonia among foals, and the total concentration of \( R_{equi} \) and concentration of virulent \( R_{equi} \) were quantified for each fecal sample. Fecal concentrations in mares of foals that subsequently developed \( R_{equi} \)-associated pneumonia and in mares of foals that did not develop pneumonia were compared at the time of each sample collection, and changes in concentrations in each group among the different sampling times were assessed.

Materials and Methods

Study population and fecal sample collection—Thoroughbred mares and their foals from a breeding farm in central Kentucky on which \( R_{equi} \)-associated pneumonia was endemic were used as the study population. Each year, the farm housed approximately 120 mares (on 250 acres). From January through April, horses were kept in stalls at night and in pastures during daylight hours. From April through December, horses were moved into stalls for 2 to 3 hours each day for feeding and for visual inspection and monitoring of rectal temperatures of foals. Generally (approx 90% of the time), a mare (or mare-foal pair) had its own stall. During the 5 years prior to initiating this study, the annual proportion of foals born at the farm that developed \( R_{equi} \)-associated pneumonia was approximately 25% to 30%. During the study period (2004 and 2005), 1 L of commercially available plasma1 that was hyperimmune against \( R_{equi} \) was transfused to all foals born on the farm within 12 hours of birth and again at 28 days for all foals still residing at the farm at that age. Only mares whose foals were born alive and resided at the farm from birth through weaning were included in the study. The rationale for this inclusion criterion was to ensure consistent monitoring for development of \( R_{equi} \)-associated pneumonia by 2 of the authors (NMS and GDM). Fecal samples were collected from mares at various time points before and after parturition as follows: approximately 2 weeks prior to the expected date of parturition, approximately 1 week prior to the expected date of parturition, the day of parturition, and 1 week after parturition. Expected parturition dates were estimated on the basis of the last breeding date.

Fecal samples were collected either from the top of a fresh fecal pile that the horse was known to have voided in the stall or by manual evacuation of feces from the rectum. Following collection, fecal samples were refrigerated at 4°C until shipped; within 48 hours of collection, samples were transported to the Equine Infectious Diseases Laboratory, Texas A&M University in insulated containers with icepacks. During 2004, fecal samples were frozen at –20°C on arrival at the Equine Infectious Diseases Laboratory and prior to culture. During 2005, quantitative bacteriologic culture of feces was performed on fresh samples on the day of arrival or the following day for samples that arrived late in the day; specimens that were cultured on the next day were maintained under refrigeration. The reason for the discrepancy in specimen handling was that sample collection was initiated in 2004 prior to receipt of funding for the project; samples initially were frozen and stored in the event that the study could not be conducted because of lack of funding. When funding was obtained, the investigators elected to freeze all fecal samples for 2004 so that they would be processed in a consistent manner.

Bacteriologic culture of \( R_{equi} \)—A 1-g aliquot of each fecal specimen was mixed thoroughly with 5 mL of PBS10 to evenly distribute the microorganisms. Quantitative bacteriologic culture of the fecal suspensions was performed by use of a modified NANAT agar medium11 that was developed by one of the authors (ST). The modified NANAT agar medium consisted of the following: boiled beef broth (300 mL), distilled water (600 mL), peptone12 (20 g), sodium chloride12 (5 g), yeast extract12 (5 g), glucose12 (5 g), sodium dichloride13 (0.2 g), sodium thiosulfate13 (1.2 g), potassium phosphate12 (2 g), sodium bicarbonate12 (2 g), and technical agar13 (15 g). The pH was adjusted to 7.2 and autoclaved at 121.6°C for 30 minutes. The medium was allowed to cool to 55°C before antimicrobials were added as follows: novobiocin1 (25 µg/mL), cycloheximide14 (40 µg/mL), nalidixic acid15 (20 µg/mL), and potassium tellurite16 (to a concentration of 3.5%). Although either beef broth or horse-meat broth can be used in this formulation, beef broth was prepared for use in this study. Approximately 1.1 kg of lean ground beef was mixed with 5 L of distilled water and incubated overnight at 4°C. The mixture was then boiled...
for 2 hours, cooled at room temperature (approx 22°C), and stored at 4°C until completely cooled. The solidified fat was removed from the surface, and broth was strained through gauze to remove residual fat and meat. The broth was then autoclaved at 121.6°C for 30 minutes and stored at 4°C.

The use of the modified NANAT agar medium effectively minimized concomitant growth of bacterial and fungal contaminants. To examine the possibility of R. equi growth inhibition on the modified NANAT agar, virulent and plasmid-cured (avirulent) R. equi strains were quantitatively cultured on the modified NANAT agar medium from R. equi minimal media. Growth of each R. equi strain appeared uninhibited on the modified NANAT agar in pure culture and after mixing with equine fecal material to mimic other potential growth-inhibiting sources.

Ten-fold serial dilutions of the fecal-PBSS suspensions were prepared and cultured in duplicate for all samples. A 100-µL volume of the fecal suspension was inoculated onto the surface of an NANAT medium agar plate and evenly dispersed by use of a sterile plate spreader. Positive and negative control specimens were cultured to ensure validity of the results. The positive control specimens consisted of pure culture virulent R. equi (American Type Culture Collection strain 33701) grown in R. equi minimal medium to approximately 10^8 CFUs/mL or 1 g of feces that was inoculated with approximately 10^8 CFUs of virulent R. equi. Ten-fold serial dilutions were performed on both of the positive control specimens, and 100 µl of the dilution containing approximately 100 CFUs of virulent R. equi/mL was plated onto separate agar plates. The negative control specimens consisted of 100 µl of R. equi minimal medium (used to make the pure cultures) or 100 µl of PBSS (used to resuspend feces for quantitative bacteriologic culture). All quantitative culture control specimens were plated on the modified NANAT agar medium used for all other samples in the study.

Control and fecal specimen culture plates were incubated at 34°C for 48 hours. For each dilution of each fecal sample, the number of CFUs of R. equi per culture plate was counted; the dilution containing 15 to 150 CFUs of R. equi/mL was plated onto separate agar plates. The next lower dilution, the plate with the lower number of CFUs was used. The number of CFUs on a plate was determined by total number of CFUs was used. The number of CFUs on a plate was divided at nor duplicate was determined and used for analysis.

Modified colony immunoblot assay—The concentration of virulent R. equi in feces was determined by use of a modified immunoblotting technique for detection of VapA. Colony immunoblotting involving a monoclonal antibody against VapA has been previously used to determine the virulence status of 3 to 10 R. equi colonies subcultured from their original agar plates. This approach was modified to allow quantification of the concentration of virulent R. equi within a background of bacterial and fungal contamination. Nitrocellulose membranes were placed onto previously incubated modified NANAT agar culture plates containing 15 to 150 CFUs of R. equi and allowed to become completely saturated. Once saturated, membranes were removed, air-dried at room temperature for 30 minutes, and baked in an oven at 100°C for 1 minute. The nitrocellulose membranes were incubated in 5% nonfat dry milk (diluted in TBSS) for 60 minutes at 37°C to block any unbound sites. The membranes were then washed 3 times in TBSS with 0.05% Tween 20. The membranes were incubated overnight at 4°C on a rocker with the monoclonal antibody (provided by ST) diluted 1:10,000 in 5% nonfat dry milk. The membranes underwent 3 washes (10 minutes each) at 37°C with fresh TBSS. Horseradish peroxidase-conjugated goat IgG fraction against mouse IgG was diluted 1:2,000 in 5% nonfat dry milk and added to the membranes, which were then incubated for 1 hour at 37°C. The membranes underwent 3 washes (10 minutes each) at 37°C with fresh TBSS, 1 wash with citrate-EDTA buffer (10mM sodium citrate and 10mM EDTA; pH, 5.0) for 5 minutes at 37°C, 1 wash with citrate-EDTA buffer and 1% dextran sulfate for 10 minutes at 37°C, and 3 washes with citrate-EDTA buffer for 5 minutes at 37°C. The substrates 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide were added to the membranes, which were then incubated for 30 minutes at 37°C. The substrate was discarded, and distilled water was added to stop development. Virulent R. equi colonies appeared blue, whereas the avirulent R. equi colonies and contaminants remained colorless. This colony immunoblotting method was validated by use of both virulent and avirulent strains of R. equi. The immunoblotting method and a previously described colony picking method were performed with each strain of R. equi grown on modified NANAT agar medium (from R. equi minimal medium). In addition, to validate proper selectivity of the monoclonal antibody against virulent R. equi isolates, plates of R. equi–negative feces that were experimentally inoculated with virulent or avirulent R. equi were also evaluated via colony immunoblotting after a nitrocellulose membrane was placed directly onto each agar plate. Confirmation that only virulent R. equi colonies yielded positive immunoblot results was made prior to implementation of this technique. Positive and negative control specimens were also included along with each batch of colony immunoblots. The positive and negative control specimens consisted of modified NANAT agar medium plates grown from pure cultures of virulent and avirulent R. equi in R. equi minimal medium, respectively. Ten-fold serial dilutions were performed on the virulent and avirulent R. equi by use of PBSS and 100 µl of the dilution containing approximately 10^8 CFUs of R. equi/mL was individually plated. All colony immunoblot control plates were incubated under the same conditions as the quantitative bacteriologic culture control plates and the fecal specimen plates. The proportion of virulent isolates in the fecal specimen plates was determined by dividing the total number of virulent colonies from both duplicate plates by the total number of R. equi colonies that were present on both duplicate plates prior to
Assessment of foals for \( R\ equi \)-associated pneumonia—Beginning in the first week of life, foals were monitored daily for abnormalities (eg, high respiratory rate or pronounced respiratory effort, or joint swelling) via visual inspection by one of the authors (GDM) and assessment of rectal temperature by farm staff; a physical examination was performed immediately if fever (rectal temperature > 38.9°C [> 102°F]) or other physical abnormalities were detected. To identify peripheral pulmonary abscesses, lung consolidation, or other abnormalities such as irregularities of the pleural surface, thoracic ultrasonography was performed at approximately 2 weeks of age and repeated at 2-week intervals until weaning (16 to 20 weeks). Additionally, foals underwent thoracic ultrasonography within 48 hours if fever or another physical abnormality was detected during daily screening. Each foal included in the study was examined ultrasonographically, as previously described.\(^{16,18} \) Foals that had ultrasonographic evidence of a peripheral pulmonary abscess or lung consolidation underwent an aseptic TBA procedure. Samples of TBA fluid were cultured to detect \( R\ equi \) via routine methods at the laboratory of the Hagyard Equine Medical Institute. For purposes of the study, foals > 2 weeks of age that had ultrasonographic evidence of pulmonary abscesses or lung consolidation and isolation of \( R\ equi \) from a TBA specimen were classified as having \( R\ equi \)-associated pneumonia. Although it was not a criterion for case definition, all isolates of \( R\ equi \) obtained from TBA specimens were sent to the Equine Infectious Disease Laboratory at Texas A&M University; the isolates were confirmed to be \( R\ equi \) and determined to be positive for the \( \text{vapA} \) gene by use of a multiplex PCR method,\(^{18} \) which also included universal bacterial primers to ensure that each DNA extraction was successful.

Data analysis—Total concentration of \( R\ equi \), concentration of virulent \( R\ equi \), and proportion of virulent \( R\ equi \) in mares of foals that subsequently developed \( R\ equi \)-associated pneumonia (hereafter referred to as the affected-foal group) and in mares of foals that did not develop pneumonia (hereafter referred to as the unaffected-foal group) were compared at each time point. Furthermore, the differences in these fecal outcomes between time points were calculated (ie, difference 1 = [value at 2 weeks prior to parturition] – [value at 1 week prior to parturition]; difference 2 = [value at 2 weeks prior to parturition] – [value on day of parturition]; difference 3 = [value at 2 weeks prior to parturition] – [value 1 week after parturition]; difference 4 = [value at 1 week prior to parturition] – [value on day of parturition]; difference 5 = [value at 1 week prior to parturition] – [value 1 week after parturition]; and difference 6 = [value on day of parturition] – [value 1 week after parturition]). Comparisons were made among the 6 calculated differences within each group and between the calculated differences in the 2 groups. Data were summarized as median values and IQ ranges for continuous data (such as concentrations or proportions of virulent organisms) and contingency tables for categoric data. Continuous variables were compared by use of the Wilcoxon rank sum test (for comparison of 2 samples) or Kruskal-Wallis test (for comparisons of > 2 samples) because these data generally appeared non-Gaussian. Categoric variables were compared by use of the \( \chi^2 \) test or, when appropriate, Fisher exact test. Because multiple comparisons were made, the adjustment method of Holm\(^{20} \) was used to retain an overall significance value of \( P < 0.05 \) when any comparison was identified as being significant. Analyses were conducted by use of commercial software.\(^{21} \)

Results

Ninety-four and 77 mares each had 1 foal that was born alive and resided at the farm from birth through weaning in 2004 and 2005, respectively; 41 mares contributed data in both years such that 130 different mares were evaluated during the 2-year study. During 2004, the 94 mares contributed 365 fecal samples (97% of a total of 376 [94 × 4] possible samples); samples were not available for 2 mares at 2 weeks prior to parturition, 4 mares at 1 week prior to parturition, 2 mares on the day of parturition, and 3 mares at 1 week after parturition. During 2005, the 77 mares contributed 286 fecal samples (93% of a total of 308 [77 × 4] possible samples); samples were not available for 4 mares at 1 week prior to parturition, 10 mares on the day of parturition, and 8 mares at 1 week after parturition. Because fecal samples were processed differently in 2004 and 2005 (ie, bacteriologic culture of frozen vs fresh samples), the effects of year on fecal shedding of \( R\ equi \) and whether the mare's foal developed \( R\ equi \)-associated pneumonia were examined. Because there were significant differences in the concentration of \( R\ equi \) and the proportion of virulent organisms in fecal samples between years (Table 1), the data were analyzed separately for 2004 and 2005. However, there were no significant differences in the distribution of the number of fecal samples per mare (as many as 4 samples/mare) that were positive for virulent \( R\ equi \) or in the prevalence of mares with diseased foals (31% [29/94 foals] in 2004 and 31% [24/77 foals] in 2005) between years.

Association of \( R\ equi \) in feces with pneumonia status of the foal among mares during 2004—Of the 94 mares included in the study during 2004, 29 (31%) had foals for which a diagnosis of \( R\ equi \)-associated pneumonia was subsequently made. An additional 11 foals had ultrasonographic evidence of pulmonary abnormalities, but \( R\ equi \) was not isolated from their TBA fluid nor were gram-positive intracellular coccobacilli evident via cytologic examination of TBA fluid; \( \beta\)-hemolytic streptococcal organisms were isolated from 7 of these 11 foals; among the remaining foals, either no organisms (2 foals) or presumed contaminants (2) were isolated. After data had been collected and evaluated, it was noted that after June 1, 2004, the proportion of foals with signs of pneumonia (ie, ultrasonographic and clinical findings that prompted a TBA procedure) was significantly (\( P < 0.001 \)) greater among the foals for which \( R\ equi \) was not isolated from TBA fluid (9/11 foals [82%]), compared with the foals for which \( R\ equi \)
was isolated from TBA fluid (5/29 foals [17%]). Overall, the age at which signs of pneumonia were detected was significantly ($P < 0.001$) greater among foals for which $R$ equi was not isolated from TBA fluid (median age, 13 weeks; IQ range, 6 to 15 weeks), compared with foals for which the organism was isolated from TBA fluid (median age, 6 weeks; IQ range, 5 to 6 weeks); this also was a post hoc finding.

For the mares that had foals with $R$ equi–associated pneumonia (the affected-foal group) and mares that had foals that were not affected with $R$ equi–associated pneumonia (the unaffected-foal group), the total concentration of $R$ equi, the concentration of virulent $R$ equi, or the proportion of virulent $R$ equi in feces did not differ significantly (Table 2). Virulent $R$ equi were detected in the feces of all mares at 1 or more time point; results of bacteriologic culture were positive for virulent $R$ equi for 1 (1%) mare at 1 time point (this mare was in the unaffected-foal group), 16 (17%) mares at 2 time points, 43 (46%) mares at 3 time points, and 34 (36%) mares at all 4 time points. There was no significant difference in the distribution of the number of fecal samples in which virulent $R$ equi were detected per mare between the affected-foal and unaffected-foal groups; 26 (90%) mares in the affected-foal group and 51 (78%) mares in the unaffected-foal group yielded virulent $R$ equi from fecal samples collected at 3 or all 4 time points.

Within each group, there were no significant differences for any of the fecal outcomes (total concentration of $R$ equi, concentration of virulent $R$ equi, or proportion of virulent $R$ equi) among the 6 pairs of differences in sampling times (eg, difference 1 = [value at 2 weeks prior to parturition] – [value at 1 week prior to parturition]; difference 2 = [value at 2 weeks prior to parturition] – [value on day of parturition], and so forth). Additionally, comparison of each of the calculated differences between the affected-foal and unaffected-foal groups revealed no significant differences.

Table 1—Median (IQ range) values for the total concentration of Rhodococcus equi, concentration of virulent $R$ equi, and proportion of virulent $R$ equi in 171 fecal samples collected from 135 mares on a farm in central Kentucky at intervals during 2004 and 2005.

<table>
<thead>
<tr>
<th>Time point to parturition</th>
<th>2004</th>
<th>2005</th>
<th>P value*</th>
<th>2004</th>
<th>2005</th>
<th>P value*</th>
<th>2004</th>
<th>2005</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks prior to parturition</td>
<td>1,525 (1,100–2,206)</td>
<td>2,450 (1,225–7,500)</td>
<td>0.004</td>
<td>160 (74–252)</td>
<td>125 (50–504)</td>
<td>1.000</td>
<td>11 (6–15)</td>
<td>6 (3–10)</td>
<td>0.022</td>
</tr>
<tr>
<td>1 week prior to parturition</td>
<td>1,225 (950–1,819)</td>
<td>2,450 (1,225–3,800)</td>
<td>&lt; 0.001</td>
<td>115 (52–211)</td>
<td>125 (51–276)</td>
<td>0.770</td>
<td>9 (5–16)</td>
<td>7 (3–10)</td>
<td>0.039</td>
</tr>
<tr>
<td>Day of parturition</td>
<td>1,262 (750–1,969)</td>
<td>2,450 (1,038–9,625)</td>
<td>&lt; 0.001</td>
<td>120 (54–226)</td>
<td>100 (25–290)</td>
<td>1.000</td>
<td>10 (3–16)</td>
<td>5 (3–8)</td>
<td>0.005</td>
</tr>
<tr>
<td>1 week after parturition</td>
<td>1,475 (762–3,000)</td>
<td>2,500 (800–10,500)</td>
<td>0.049</td>
<td>70 (0–198)</td>
<td>98 (25–502)</td>
<td>0.770</td>
<td>5 (0–12)</td>
<td>4 (1–8)</td>
<td>0.770</td>
</tr>
</tbody>
</table>

*P values were adjusted for multiple comparison; results of significance testing were similar without adjustment (ie, classification of any comparison as significant was not altered by adjustment, only the magnitude of the associated $P$ value was changed.)

Table 2—Median (IQ range) values for the total concentration of $R$ equi, concentration of virulent $R$ equi, and proportion of virulent $R$ equi in fecal samples collected from 94 mares (by disease status of their foals) on a farm in central Kentucky at intervals during 2004.

<table>
<thead>
<tr>
<th>Time point to parturition</th>
<th>Affected-foal group*</th>
<th>Unaffected-foal group*</th>
<th>P value</th>
<th>Affected-foal group*</th>
<th>Unaffected-foal group*</th>
<th>P value</th>
<th>Affected-foal group†</th>
<th>Unaffected-foal group†</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks prior to parturition</td>
<td>1,525 (1,150–2,144)</td>
<td>1,525 (1,019–2,225)</td>
<td>0.788</td>
<td>170 (90–215)</td>
<td>158 (69–260)</td>
<td>0.875</td>
<td>11 (6–15)</td>
<td>11 (6–15)</td>
<td>0.993</td>
</tr>
<tr>
<td>1 week prior to parturition</td>
<td>1,175 (775–1,450)</td>
<td>1,300 (975–1,825)</td>
<td>0.319</td>
<td>100 (60–180)</td>
<td>125 (50–240)</td>
<td>0.525</td>
<td>11 (5–18)</td>
<td>9 (5–15)</td>
<td>0.473</td>
</tr>
<tr>
<td>Day of parturition</td>
<td>1,225 (794–1,556)</td>
<td>1,350 (750–2,156)</td>
<td>0.363</td>
<td>110 (55–176)</td>
<td>135 (49–264)</td>
<td>0.554</td>
<td>10 (4–16)</td>
<td>10 (3–16)</td>
<td>0.100</td>
</tr>
<tr>
<td>1 week after parturition</td>
<td>1,912 (988–3,106)</td>
<td>1,450 (562–2,800)</td>
<td>0.429</td>
<td>48 (0–141)</td>
<td>125 (0–208)</td>
<td>0.420</td>
<td>5 (1–10)</td>
<td>5 (0–12)</td>
<td>0.990</td>
</tr>
</tbody>
</table>

*Affected-foal group = Mares that had a foal that developed $R$ equi–associated pneumonia (n = 29). †Unaffected-foal group = Mares that had a foal that did not develop $R$ equi–associated pneumonia (n = 65).
9 weeks), compared with foals for which the organism was isolated from TBA fluid (median age, 5 weeks; IQ range, 5 to 6 weeks), the difference was not significant \((P = 0.065)\). As stated for the data for 2004, the 2 aforementioned comparisons were not a priori objectives of the present study.

For mares in the affected-foal group and mares in the unaffected-foal group, the total concentration of \(R\ equi\), the concentration of virulent \(R\ equi\), or the proportion of virulent \(R\ equi\) did not differ significantly (Table 3). Virulent \(R\ equi\) isolates were detected in the feces of all mares at 1 or more time points; results of bacteriologic culture were positive for \(R\ equi\) for 2 (3%) mares at 1 time point (1 mare each in the affected and unaffected groups), 11 (14%) mares at 2 time points, 36 (47%) mares at 3 time points, and 28 (36%) mares at all 4 time points. There was no significant difference in the distribution of the number of fecal samples in which virulent \(R\ equi\) were detected per mare between the affected-foal and unaffected-foal groups; 21 (91%) mares in the affected-foal group and 43 (80%) mares in the unaffected-foal group yielded virulent \(R\ equi\) from fecal samples at 3 or all 4 time points.

Within each group, there were no significant differences for any of the fecal outcomes (total concentration of \(R\ equi\), concentration of virulent \(R\ equi\), or proportion of virulent \(R\ equi\)) among the 6 pairs of differences in sampling times. Additionally, comparison of each of the calculated differences between the affected-foal and unaffected-foal groups revealed no significant differences.

Mares with foals born in both years of the study—Forty-one mares gave birth to a foal during both 2004 and 2005. Only 3 of 41 mares had foals that developed \(R\ equi\)-associated pneumonia during both years, whereas there were 8 mares that had affected foals during 2004 but not 2005, 12 mares that had foals affected during 2005 but not 2004, and 18 mares with foals that were unaffected in 2004 and 2005. Thus, there appeared to be no evidence that certain mares were more likely than other mares to have foals that would develop \(R\ equi\)-associated pneumonia.

**Discussion**

*Rhodococcus equi* is a bacterial pathogen that causes disease in foals worldwide. This bacterium can be isolated from many sources including feces, soil, and TBA fluid. Unfortunately, much is still unknown regarding the transmission of \(R\ equi\). The primary aim of the study reported here was to determine whether mares were a potential source of \(R\ equi\) for their foals. Although disease caused by \(R\ equi\) is not commonly diagnosed in adult horses, horses excrete varying fecal concentrations of virulent \(R\ equi\) into their environment. Foals exposed to this contaminated environment are thus likely to become exposed to virulent \(R\ equi\) from birth, and epidemiologic evidence indicates that foals may be infected during the first few days of life. The route of \(R\ equi\) infection for foals is unknown but is presumed to involve inhalation of organisms from the environment. Because it is common for foals to ingest small quantities of fresh feces passed by their dams, it is also possible that foals might become infected as a result of coprophagia. Irrespective of the route of infection, results of the present study indicate that foals at breeding farms may be exposed from the time of birth to virulent \(R\ equi\) from their dams and other mares.

An important finding in the present study was that virulent isolates of \(R\ equi\) were identified in the feces of all mares evaluated. To the authors’ knowledge, a similar finding has not been previously reported. Although contamination of fecal specimens either prior to or during collection, or after collection in the laboratory, could explain this finding, we do not believe this to be the case for several reasons. With regard to contamination of fecal specimens during collection, the greatest number of \(R\ equi\) is recovered from the soil surface and isolation of \(R\ equi\) from the air has been reported to increase on dry, windy days. Thus, movement of dust during a windy day was a potential contaminating source of the organism for our samples. Most fecal samples were collected from the top of a fresh fecal pile (ie, feces that were not in contact with the stall to minimize environmental contamination), but some were collected from the rectum by manual evacuation. Given that nearly all samples were positive for \(R\ equi\), one would have to assume that there was sufficient wind and dust on all days that samples were collected during the winter and spring to contaminate nearly all samples collected from stalls inside barns. Concentrations of \(R\ equi\) cultured from dams’ feces ranged from >10^2 to >10^3 CFUs/g, which is consistent with past reports. If contamination of fecal samples was the

<table>
<thead>
<tr>
<th>Time point to parturition</th>
<th>Affected-foal group*</th>
<th>Unaffected-foal group†</th>
<th>(P) value</th>
<th>Affected-foal group*</th>
<th>Unaffected-foal group†</th>
<th>(P) value</th>
<th>Affected-foal group*</th>
<th>Unaffected-foal group†</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks prior to parturition</td>
<td>1,812 (1,025–6,375)</td>
<td>2,675 (1,475–7,500)</td>
<td>0.189</td>
<td>75 (50–312)</td>
<td>150 (73–748)</td>
<td>0.239</td>
<td>6 (4–9)</td>
<td>6 (3–12)</td>
<td>0.926</td>
</tr>
<tr>
<td>1 week prior to parturition</td>
<td>2,050 (988–3,212)</td>
<td>2,525 (1,512–3,975)</td>
<td>0.370</td>
<td>125 (50–275)</td>
<td>150 (75–367)</td>
<td>0.552</td>
<td>6 (4–9)</td>
<td>7 (3–10)</td>
<td>0.777</td>
</tr>
<tr>
<td>Day of parturition</td>
<td>2,250 (1,588–4,088)</td>
<td>2,962 (984–10,312)</td>
<td>0.501</td>
<td>101 (25–239)</td>
<td>100 (25–565)</td>
<td>0.756</td>
<td>5 (2–7)</td>
<td>5 (3–9)</td>
<td>0.771</td>
</tr>
<tr>
<td>1 week after parturition</td>
<td>1,625 (788–14,125)</td>
<td>2,600 (1,401–10,188)</td>
<td>0.674</td>
<td>125 (6–627)</td>
<td>87 (25–438)</td>
<td>0.873</td>
<td>4 (0–8)</td>
<td>4 (1–7)</td>
<td>0.419</td>
</tr>
</tbody>
</table>

*Affected-foal group = Mares that had a foal that developed \(R\ equi\)-associated pneumonia \((n = 53)\). Unaffected-foal group = Mares that had a foal that did not develop \(R\ equi\)-associated pneumonia \((n = 24)\).
predominating explanation for our findings, we might not have expected to find similar fecal concentrations of R equi as those previously reported. Nevertheless, contamination of the voided feces by isolates of R equi that were in soil, bedding, or in the air cannot be excluded. With regard to contamination of fecal samples at the laboratory, samples were individually packaged and shipped in sealed sacks. In the laboratory, negative control plates were used during each culture procedure such that contamination of reagents and instruments with virulent R equi would have been detected.

Other explanations for the high prevalence of virulent R equi in fecal specimens exist. Sequential sample collection provided increased opportunity to identify R equi in feces. Use of a modified NANAT medium improved our ability to culture R equi relative to that achieved by use of other media, including standard NANAT medium (data not shown). The high prevalence of R equi in feces is consistent with at least 1 previous report from Australia. To the authors’ knowledge, this is the first report of a method for quantifying the concentration of virulent organisms in fecal samples. Previously, the proportion of virulent isolates in fecal samples was estimated by selectively subculturing ≤ 10 colonies selected at random from plates and testing these specimens for expression of VapA via immunoblotting. With the modified colony immunoblot protocol, placement of nitrocellulose membranes directly on the culture plates allowed for evaluation of the virulence status of all R equi isolates on a plate, rather than just a portion. Conceivably, the finding of a higher prevalence of fecal shedding of virulent isolates among mares in our study may be a function of improved culture techniques and the use of the complete plate colony immunoblot method used. Interestingly, the proportion of concentrations of virulent isolates in the study of this report was similar in magnitude to the proportions of virulent isolates among isolates subcultured from fecal samples in previous studies. Negative and positive control plates were also blotted along with the fecal specimen culture plates to ensure that the colony immunoblot method was properly controlled.

There are a number of limitations of the present study. Data were obtained from 1 farm, and it is unclear to what extent these results would be representative of other farms with recurrent episodes of R equi–associated pneumonia among foals. Additionally, it would be interesting to know whether the prevalence and concentration of fecal excretion of virulent R equi vary among farms on which foals develop R equi–associated pneumonia and unaffected farms. Results of such studies would help elucidate the association between R equi shed in the feces of mares at breeding farms and the R equi–associated pneumonia status of foals at those farms. Our rationale for designing a study in which only 1 farm was examined was manifold. First, the primary objective of the study was to determine whether mares were a source of R equi for their foals, which required intensive data collection and monitoring. Thus, the study was labor intensive, and considerable effort was required to accurately collect and transport samples and determine the disease status of individual foals. Finding veterinarians and farms willing and able to collaborate would have been challenging. Second, even if additional farms could have been identified, completion of this investigation in a more comprehensive group of farms would have required a budget beyond the resources of available funding sources. Third, there were no fecal concentration data from mares at breeding farms in North America on which to base sampling estimates. However, results of the present study provide important data with which other investigators may conduct studies to confirm the external validity of our results and test additional hypotheses regarding fecal concentrations of virulent R equi among mares and the incidence of R equi–associated pneumonia among foals.

Four fecal samples were not available from all mares in the present study. Because parturition dates were estimated on the basis of the last breeding date, some mares gave birth earlier than expected; thus, samples were not collected from all mares at each time point. In other instances, fecal samples were not collected on the day of or at 1 week after parturition because of the absence of the technician who was responsible for collecting specimens. The impact of this incomplete sample collection was minimal in light of the fact that > 95% of samples were collected and the finding that virulent R equi was detected in feces from all mares.

Another limitation of the present study was the possible misclassification of foals. Only foals that yielded R equi from their TBA fluid were classified as having R equi–associated pneumonia. Although the sensitivity of bacteriologic culture is generally high (57% to 100%), false-negative results may occur. Among foals in which R equi–associated pneumonia was confirmed after necropsy, analysis of TBA fluid for R equi as a means of diagnosis of R equi infection has been reported to be 100% sensitive. Conceivably, false-negative results might be more likely to occur during the early stages of disease. Because foals included in our study were systematically screened for disease development beginning in the first week of life, signs of pneumonia were detected during the early stages of the disease. In the present study, TBA fluid analysis yielded negative results for R equi in approximately 28% of foals that had ultrasonographic findings of pulmonary lesions. In some of these foals, R equi was most likely not the cause of the lung lesions, particularly when β-hemolytic streptococcal organisms were isolated from the TBA fluid. However, it is also possible that some foals that had pulmonary lesions but no detectable R equi in TBA fluid were truly infected with R equi and that the results of bacterial cultures of TBA fluid were false negative. To assess the potential impact of this misclassification on results of the present study, data were analyzed post hoc such that all foals with clinical and ultrasonographic evidence of pneumonia (irrespective of results of bacteriologic culture of TBA fluid) were considered to have R equi–associated pneumonia; results of those analyses yielded similar results to those obtained initially. Thus, misclassification of foals with false-negative results of TBA fluid assessment did not appear to bias the results of our study. Moreover, compared with foals that underwent a TBA procedure and
provided fluid from which \( R\ equi \) was isolated, foals that underwent a TBA procedure and provided fluid from which \( R\ equi \) was not isolated were older (the difference was significant in 2004 but not in 2005) and developed evidence of pneumonia later in the year; these findings support that those foals were appropriately classified as different from the \( R\ equi \)-positive foals.

In 2004, collected fecal specimens were refrigerated for transportation to the Equine Infectious Diseases Laboratory at Texas A& M University and immediately frozen at \(-20^\circ\)C on arrival. It was speculated that there would be a loss in the numbers of \( R\ equi \) if the feces were frozen prior to being cultured, but the rate of that loss was unknown. Experimentally, we have determined that freezing of fecal specimens at \(-20^\circ\)C results in approximately a 0.5 to 1.0 \( \log_{10} \) reduction in growth of \( R\ equi \). To avoid reduction in bacterial numbers as a result of freezing, the fecal samples in 2005 were cultured on arrival at the Equine Infectious Diseases Laboratory. Compared with samples collected in 2004, a larger number of contaminants were detected during bacteriologic culture of samples collected in 2005, likely because the latter samples had not been frozen prior to culture; although freezing of fecal samples reduced the growth of \( R\ equi \), it also reduced the growth of contaminants.

On the basis of a previous report, the dilution that provided 15 to 150 CFUs of \( R\ equi \)/culture plate was used for quantitative bacterial culture and immunoblotting. Because \( < 15 \) CFUs of \( R\ equi \) were occasionally isolated from a specimen for which the plates prepared with the next highest dilution had \( > 150 \) CFUs, continuous variables (eg, the proportion of virulent isolates) included some outliers. This was more of an issue with the data from 2004 because fecal specimens were frozen at \(-20^\circ\)C and the resultant fecal concentrations of \( R\ equi \) were comparatively lower. The Wilcoxon rank sum and the Kruskal-Wallis tests were used for analyses because they are less sensitive to the effects of outliers and the non-Gaussian distribution of data. Analysis of the data following logit or other transformations to render them more Gaussian in distribution did not alter results of our study. Nevertheless, the fact that the denominator used for calculation of the proportion of virulent organisms was generally smaller in 2004 than in 2005 meant that this proportion was more sensitive to the presence or absence of a given colony (ie, the difference between 4 and 5 immunoblot-positive colonies was more influential on the estimated proportion of virulent organisms when the denominator was 15 than when it was 100). It is probable that the significant difference in the proportion of virulent isolates between years was attributable to the fact that there were more plates with small total numbers of \( R\ equi \) colonies in 2004 than in 2005. Alternatively, it is possible that virulent isolates were less affected by freezing at \(-20^\circ\)C than were avirulent isolates. To the authors’ knowledge, experimental evidence to support this hypothesis is lacking.

Comparisons of the concentrations and proportions of \( R\ equi \) between years assumed independence of samples and ignored the fact that some samples were correlated (ie, some mares contributed samples in both years). We believe the impact of this assumption is minimal for several reasons. Nearly all samples from a given mare at a given time point had lower \( R\ equi \) concentrations in 2004 than in 2005 (data not shown). By ignoring the correlation in the data, we underestimated the variance of data used for significance testing. Although we may have overestimated the number of significant differences or the magnitude of significance of these differences, samples were processed differently between years and exploratory data analysis between years supported our approach of analyzing data separately from each of the 2 years. Although it does not speak directly to this issue, magnitudes of \( P \) values for the between-year comparisons were all adjusted (inflated) for the 12 comparisons that were made.

The objective of the present study was to determine whether mares were a possible source of infection for their foals; however, exposure of foals to virulent \( R\ equi \) was not directly assessed. Serologic assessment of exposure to virulent \( R\ equi \) in foals is problematic because of poor sensitivity and specificity of available tests. Use of the methods described in this report to evaluate fecal specimens might yield evidence of exposure to virulent \( R\ equi \); such monitoring was beyond the scope of our study.

Results of the study of this report are important because they indicate that feces from all mares at a breeding farm that were known to have recurrent episodes of \( R\ equi \)-associated pneumonia among foals contained virulent \( R\ equi \) during the perinatal period. These virulent isolates were a potential source of infection for susceptible foals. It would be important to substantiate these findings at other farms and compare findings of fecal concentrations of \( R\ equi \) from mares on farms with recurrent episodes of \( R\ equi \)-associated pneumonia with those of foals and mares on farms without such history. The method of quantifying the concentration of virulent \( R\ equi \) in feces described in this report may be useful to other investigators for use in studies involving feces or other specimens, such as soils or tissues.

a. Anti-\( Rhodococcus\ equi\) antibody, Lake Immunogenics, Ontario, NY.
b. PBS solution (pH, 7.2), Invitrogen Co, Carlsbad, Calif.
c. Peptone from Glycine max (soybean), Type IV, powder, Sigma Chemical Co, St Louis, Mo.
d. Sodium chloride, American Chemical Society certified reagent, Sigma Chemical Co, St Louis, Mo.
e. Yeast extract, Fluka by Sigma Chemical Co, St Louis, Mo.
f. D-(+)-glucose, anhydrous, Sigma Chemical Co, St Louis, Mo.
g. Sodium dithionite, > 85% (as determined by redox titration), Fluka by Sigma Chemical Co, St Louis, Mo.
h. Sodium thioglycollate, 99%, Sigma Chemical Co, St Louis, Mo.
i. Potassium phosphate dibasic powder, Sigma Chemical Co, St Louis, Mo.
j. Sodium bicarbonate, Sigma Chemical Co, St Louis, Mo.
k. Technical agar, Difco by BD Biosciences Co, San Jose, Calif.
l. Novobiocin sodium salt minimum (90%), Sigma Chemical Co, St Louis, Mo.
m. Cycloheximide minimum (94%), Sigma Chemical Co, St Louis, Mo.
n. Nalidixic acid sodium salt, Sigma Chemical Co, St Louis, Mo.
o. Potassium tellurite powder, Sigma Chemical Co, St Louis, Mo.
p. Grimm MB: Evaluation of mares as a source of Rhodococcus equi for their foals using quantitative culture and a colony immunoblot assay. MS thesis, Department of Large Animal Clinical Sciences, Texas A&M University, College Station, Tex, 2006.
q. Nitoscellulose membranes (pore size 0.45 µm), Bio-Rad Laboratories, Hercules, Calif.