Use of Serial Quantitative PCR of the *vapA* Gene of *Rhodococcus equi* in Feces for Early Detection of *R. equi* Pneumonia in Foals

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Background: Current screening tests for *Rhodococcus equi* pneumonia in foals lack adequate accuracy for clinical use. Real-time, quantitative PCR (qPCR) for virulent *R. equi* in feces has not been systematically evaluated as a screening test.

Objective: The objective of this study was to evaluate the accuracy of qPCR for *vapA* in serially collected fecal samples as a screening test for *R. equi* pneumonia in foals.

Animals: One hundred and twenty-five foals born in 2011 at a ranch in Texas.

Methods: Fecal samples were collected concurrently with thoracic ultrasonography (TUS) screening examinations at ages 3, 5, and 7 weeks. Affected (pneumonic) foals (n = 25) were matched by age and date-of-birth to unaffected (n = 25) and subclinical (ie, having thoracic TUS lesions but no clinical signs of pneumonia) foals (n = 75). DNA was extracted from feces using commercial kits and concentration of virulent *R. equi* in feces was determined by qPCR.

Results: Subsequently affected foals had significantly greater concentrations of *vapA* in feces than foals that did not develop pneumonia (unaffected and subclinical foals) at 5 and 7 weeks of age. Accuracy of fecal qPCR, however, was poor as a screening test to differentiate foals that would develop clinical signs of pneumonia from those that would remain free of clinical signs (including foals with subclinical pulmonary lesions attributed to *R. equi*) using receiver operating characteristic (ROC) methods.

Conclusions and Clinical Importance: In the population studied, serial qPCR on feces lacked adequate accuracy as a screening test for clinical *R. equi* foal pneumonia.

Key words: Feces; Horse; Pneumonia; *Rhodococcus equi*.

*R*hodococcus equi is a gram-positive, facultative, intracellular bacterium that is a common cause of clinical pneumonia in foals between 3 weeks and 5 months of age.[1,2] Both virulent and avirulent biotypes of *R. equi* have been identified. The presence of an 85–90-kb plasmid that encodes the virulence associated protein A (Vap A) in the *vapA* gene is required for *R. equi* to cause disease in foals.[3] Virulent isolates can thus be identified using polymerase chain reaction (PCR) to detect the *vapA* gene.[4–6]

Because of the insidious progression of infection to severe clinical signs, early and accurate diagnosis of foals with *R. equi* pneumonia is important. A definitive diagnosis is based on bacterial culture of *R. equi* or PCR

Abbreviations:

qPCR	quantitative polymerase chain reaction
ROC	receiver operator characteristic
TBA	tracheobronchial aspirate
TMD	total maximal diameter
TUS	thoracic ultrasound

amplification of *R. equi*-specific DNA from a tracheobronchial aspirate (TBA), in combination with cytological evidence of sepsis in the TBA from a foal with clinical signs of pneumonia.[2] Currently, veterinary practitioners commonly use several screening methods to try to identify foals that will develop *R. equi* pneumonia based on the rationale that earlier intervention will lead to greater therapeutic success and shorter duration of treatment. In the authors' experience, the most widely adopted approach to screening has been sequential thoracic ultrasonography (TUS) to detect abscess formation or consolidation of the peripheral lung because TUS is highly sensitive for detecting abscesses in the periphery of the lung and is easily (relative to other thoracic imaging methods) performed at farms.[2,7–9]

Use of TUS screening has revealed that many foals with findings consistent with *R. equi* infections will not develop clinical signs of pneumonia.[10,11] Even when only treating foals with larger pulmonary lesions (>200 mm total maximum diameter), serial thoracic ultrasonography performed with good sensitivity (89%) but poor specificity (62%) for the prediction of onset of clinical *R. equi* pneumonia.[11] These data indicate that TUS for screening for *R. equi* pneumonia would result in overuse of antimicrobials when foals with positive results of screening are treated with macrolides (+/− rifampin), the preferred treatment for *R. equi* pneumonia. Treating more foals with macrolides would result

in increased prevalence of adverse adverse effects in foals and their dams and increased costs for treatments, and also can contribute to emergence of bacterial resistance.[12,13]

A screening tool that can accurately predict which foals will develop *R. equi* pneumonia remains elusive. Real-time, quantitative PCR (qPCR) assays have been developed that can accurately quantify the number of virulent *R. equi* in samples.[4,6] Evidence exists that foals affected with *R. equi* pneumonia shed significantly more virulent *R. equi* in feces than unaffected foals, including foals that have subclinical pneumonia,[14,15] and that qPCR testing of feces for *vapA* can be accurate for diagnosis of *R. equi* pneumonia.[15] These findings indicate that qPCR testing of feces for *vapA* might be useful as a screening test for *R. equi* pneumonia. Thus, the purpose of this study was to evaluate use of qPCR testing of serially collected fecal samples as a screening test for development of clinical signs of *R. equi* pneumonia in foals, using a convenience sample of fecal specimens from foals with known case outcomes. We hypothesized that foals affected with *R. equi* pneumonia would shed significantly more virulent *R. equi* in feces before onset of clinical signs than foals that remained subclinical or unaffected. We further hypothesized that qPCR for *vapA* could be used to differentiate foals that would develop clinical signs of pneumonia from those that would remain free of clinical signs (including foals with subclinical pulmonary lesions attributed to *R. equi*).

Material and Methods

Study Population

The study was performed using stored feces obtained with consent from foals born in 2011 at a breeding facility in north-central Texas that were collected in conjunction with a previous study to evaluate the accuracy of hematology or TUS as screening tests for early detection of *R. equi* pneumonia. Protocols for this study were approved by the Clinical Research Review Committee (CRRC Protocol 10–12) of the College of Veterinary Medicine & Biomedical Sciences, Texas A&M University; at the time the samples were collected for this study, research involving client-owned animals at Texas A&M University was not under the purview of Texas A&M University's Institutional Animal Care and Use Committee.

As a result of our previous study,[11] foals that were followed through weaning were classified as affected with *R. equi* pneumonia, subclinical, or unaffected (healthy controls). Foals defined as affected had clinical signs suggestive of pneumonia (fever, lethargy, depression, cough, nasal discharge, polysynovitis, tachypnea, increased respiratory effort, respiratory distress, or a tracheal rattle), microbiologic isolation of *R. equi* from a TBA, cytologic evidence of sepsis of TBA fluid, and sonographically visible pulmonary consolidations or abscesses. The TBA's were collected transendoscopically on the first day of apparent clinical signs of pneumonia and submitted for cytology and aerobic culture. Foals defined as subclinical had lung lesions (consolidation or abscess formation) identified by TUS but did not develop clinical signs of pneumonia from birth through weaning. Those performing diagnosis and treatment of affected foals were blind to the results of the TUS and hematologic screening; intervention was not implemented for any foals in this study unless they developed clinical signs of pneumonia. Unaffected foals were defined as those having

neither lesions detectable by TUS nor clinical signs of pneumonia from birth through weaning. Of the 270 foals born on the farm during the 2011 breeding season, 46 foals (17%) were affected, 170 foals (63%) were subclinical, and 54 foals (20%) were unaffected.

Each of the 270 foals born during 2011 underwent TUS biweekly from 3 to 16 weeks of age. These ages were selected on the age at onset of *R. equi* pneumonia and reported procedures for TUS screening.[8,9] The total maximal diameter (TMD) of all pulmonary consolidations was recorded for each TUS examination. On the day of each TUS, a fecal sample was collected (if available), shipped chilled overnight to the Equine Infectious Disease Laboratory at Texas A&M University, and stored frozen at $-80°C$.

Sample Selection

A sample size of 125 was selected for this project on the basis of funding available to conduct the project and the number of sequential fecal samples available. From the 46 affected foals, 25 foals were selected randomly that had 3 sequential samples collected beginning with the first biweekly sample (ie, sample obtained at 3 weeks of age). The random selection was performed in 2 stages. In the first stage, the list of foals was arranged in chronological order of birthdate, and divided into 23 blocks of 2 foals. Using computerized software, we generated a list of 23 random selections of the 1 second or 2 second. For each sequential pair, whichever number was randomly generated for the corresponding pair was used to select the foal. For the remaining 2 foals, we generated a random order of numbers from 1 to 46 and the first 2 numbers that were not for foals already selected were used to select the remaining 2 foals.

For each affected foal, 1 foal born within 2 weeks of the index case and for which the first 3 sequential fecal samples were collected was selected from each of the following groups: (1) unaffected foals; (2) subclinical foals with TMD <200 mm; (3) subclinical foals with TMD 200–400 mm; and (4) subclinical foals with TMD >400 mm. Thus, for each affected foal there were fecal samples collected from 4 other foals of approximately the same age. Because we were only able to identify 24 subclinical foals with TMD of 200–400 mm, we identified an additional foal that had TMD <200 mm.

Fecal DNA Extractions

Frozen fecal samples were thawed and 100 mg of feces were aliquoted into a tube containing 1 mL of a buffer solution[a] and 50 mg of sterile DNase free 0.1- and 0.5-mm silica zirconium beads. Samples were then run through a bead mill homogenizer[b] for three 45-second cycles at a speed of 6.5 m/second and incubated in a water bath at 95°C for 5 minutes. After the incubation period, DNA was extracted using a commercially available kit according to the manufacturer's recommendations,[c] and eluted DNA was frozen at $-80°C$ in cryotubes labeled with identifiers and dates until analyzed. As an extraction control, feces from a mare known to contain virulent *R. equi* were extracted as described during each extraction.

PCR Assay

Real-time, qPCR was performed as previously described.[4] Briefly, frozen DNA samples were allowed to thaw to room temperature, and, for each reaction, 2 μL of DNA was added to 5 μL Taqman® probe,[d] 2.5 μL RNase-free water, and 0.5 μL of a custom premix,[e] such that a total of 10 μL was used for each reaction. For purposes of absolute quantification, a standard dilution curve was created. The purified plasmid (pGEX-2T; 4,948 bp) was

obtained from an *E. coli* clone containing an *R. equi* strain 103+ plasmid vector with the *vapA* gene (570 bp) that was generously provided by Dr. Steeve Giguère, University of Georgia. Plasmid DNA was extracted using a commercially available kit[f] and the concentration was determined spectrophotometrically. Ten-fold serial dilutions were made of plasmid DNA (approximate range, 10^1–10^9 copies of pGEX-2T/μg) in nuclease-free water. Plasmid DNA strands were processed in duplicate using real-time, qPCR assay. A standard curve was constructed using linear regression analysis of the $\log_{10}$ quantity of pGEX-2T copies per sample and the corresponding C_T values.

Data Analysis

Data were analyzed using S-PLUS statistical software.[g] For descriptive purposes, plots and summary statistics (including 95% confidence intervals) were used. The concentrations of *vapA* in feces were compared among the 5 groups at each of the 3 individual ages of sampling using a Kruskal–Wallis test; comparisons at each age between affected (ie, pneumonic foals) and all other foals (ie, subclinical foals and unaffected foals) were made using the Wilcoxon rank-sum test (WRST). Comparisons of categorical variables between or among groups were performed using chi-squared tests (CST). A significance level of $P < .05$ was used for analyses.

Longitudinal data (ie, sequential fecal concentrations of *vapA*) were analyzed using linear mixed-effects models to simultaneously examine the associations of age and study group on fecal concentration of *vapA* copy number. For mixed-effects modeling, *vapA* concentrations were transformed to $\log_{10}(x_{ij}+1)$ where x_{ij} was the fecal concentration of *vapA* copy number per g of feces for the ith foal at the jth sampling time. This transformation was used because many of the observed values were 0 and it was needed to meet assumptions of modeling. For mixed-effects modeling, the transformed fecal concentration was the dependent variable, sample-time, disease status and their interactions were modeled as fixed effects, and foal was modeled as a random effect to account for repeated measures. Results of qPCR for fecal samples collected after foals had been diagnosed with and treated for *R. equi* pneumonia were excluded because treatment with macrolides can reduce fecal concentrations of *R. equi* in foals.[16] Model fit was assessed using diagnostic residual plots. A value of $P < .05$ was considered significant.

Receiver operator characteristics (ROC) curves were plotted using the pROC procedure.[17] Optimal cut-points were determined using the method of Youden.[18]

Results

All affected foals recovered and all foals survived through weaning. Of the 25 affected foals, 10 developed clinical signs after 5 weeks of age (2nd sample collection) but before 7 weeks of age (3rd sample collection) and 15 foals developed clinical signs after 7 weeks of age but before 9 weeks of age (ie, after the 3rd sample). Fecal concentrations of *vapA* (ie, virulent *R. equi*) did not differ significantly among the 5 study groups at 3 weeks of age (Table 1). At 3 weeks of age, there also was no significant ($P = .648$) difference between the concentration of *vapA* in feces of the affected foals (median, 9 copies/g; range 0–1,318 copies/g) and all other foals considered as a single group (median, 7 copies/g; range 0–1,552 copies/g).

At 5 weeks of age, although fecal concentrations of *vapA* tended to be higher among affected foals than foals in other groups, there was no significant difference ($P = .195$) in concentrations among the various groups (Table 1). When comparing affected foals to all other foals, however, the fecal concentrations of *vapA* at 5 weeks of age were significantly ($P = .046$) higher for the affected foals (median, 36 copies/g; range 0–24,822 copies/g) than for the other foals (median, 7 copies/g; range 0–1,068 copies/g). Moreover, the difference in concentrations was even larger in magnitude when comparing those foals (n = 10 foals) that developed pneumonia between 5 and 7 weeks of age (median, 54 copies/g; range 1–24,822 copies/g) to all other foals (n = 115 foals; median, 7 copies/g; range 0–1,449 copies/g). The group of 115 other foals included the 15 foals that developed *R. equi* pneumonia during the 2-week interval after 7 weeks, but before 9 weeks of age.

At 7 weeks of age, although fecal concentrations of *vapA* tended to be higher among affected foals than foals in other groups, there was no significant ($P = .174$) difference in concentrations among the groups (Table 1). When comparing affected foals to all other foals, however, the fecal concentrations of *vapA* at 7 weeks of age were significantly ($P = .030$) higher for the affected foals (median, 397 copies/g; range, 0–10,869 copies/g) than for all other foals (median, 12 copies/g; range 0–3,301 copies/g). This comparison was repeated excluding the 10 foals that developed *R. equi* pneumonia before the collection of the third sample (ie, after 5 weeks but before 7 weeks of age). The concentration of fecal *vapA* was significantly ($P = .007$) greater among foals that subsequently developed pneumonia (n = 15; median, 446 copies/g; range 0–10,869 copies/g) than among all other foals that remained free of clinical signs (n = 100; median, 12 copies/g; range, 0–3,301 copies/g).

Mixed-effects modeling indicated that there were significant effects of age that depended on the development

Table 1. Median (range) of fecal concentrations (copy numbers of *vapA*/g of feces) of foals by study group and sample. Samples 1, 2, and 3 were collected at 3, 5, and 7 weeks of age, respectively. TMD = Total maximal diameter. *P* values are derived from the Kruskal–Wallis test comparing concentrations among groups within a sample-time (ie, comparisons of group within a column).

Group	Sample 1	Sample 2	Sample 3
Unaffected N = 25	9 (0–15,552)	12 (0–420)	25 (0–3,301)
Subclinical <200 mm TMD N = 26	11 (0–199)	4 (0–1068)	10 (0–2,113)
Subclinical 200–400 mm TMD N = 24	17 (0–571)	21 (0–212)	2 (0–1,182)
Subclinical 400 mm TMD N = 25	0 (0–1,276)	0 (0–878)	16 (0–726)
Rhodococcus equi pneumonia N = 25	9 (0–1,318)	36 (0–24,822)	397 (0–10,869)
P value	.6355	.1950	.1740

of subsequent pneumonia. At 3 weeks of age (initial sampling), there was no significant difference in the fecal concentrations of *vapA* between affected foals and all other foals (Fig 1). Foals that subsequently developed pneumonia had significantly higher concentrations of *vapA* in feces at 5 weeks ($P = .046$) and 7 weeks ($P = .005$) of age relative to those at 3 weeks of age; however, the fecal concentration of *vapA* in nonpneumonic (unaffected and subclinical) foals did not increase significantly over time (Fig 1).

Receiver operating characteristic methods were used to analyze the accuracy of fecal qPCR as a screening test at 3, 5, and 7 weeks of age. When the fecal concentration of *vapA* in feces was compared between affected foals (n = 25) and all other foals (n = 100), the 95% confidence intervals for the areas under the ROC curves included 50% for each age (Table 2), indicating that fecal qPCR was not useful for screening for *R. equi* pneumonia.

Foals that developed pneumonia during the 2-week interval after the sample was collected were compared to foals that did not develop pneumonia during that interval using ROC methods. The 10 affected foals (n = 10) that developed *R. equi* pneumonia between 5 and 7 weeks of age were compared to all other foals (n = 115, including 15 foals that developed *R. equi* pneumonia after 7 weeks of age). Although the 95% confidence interval for the area under the ROC curve excluded 50% (Table 2), the optimal cut-point had a specificity <43% (Fig 2). Moreover, the threshold concentration was essentially 1 copy of *vapA*/g of feces, indicating that quantitative PCR had no advantage over

Table 2. Sample 1, 2 and 3 correspond to 3, 5 and 7 weeks of age, respectively. Area under the ROC curve (AUC) and 95% confidence interval (95% CI) for fecal concentration of PCR for predicting subsequent pneumonia caused by *R. equi*.

Sample-Time	N Cases	N Controls	AUC (95% CI)
1	25	100	52.9% (39.4–66.4)
2	25	100	62.6% (49.1–76.2)
2[a]	10	115	72.9% (56–89.7)
3	25	100	64.7% (49.2–80.3)
3[b]	15	100	71.3% (53.8–88.9)

[a]Cases are only foals that developed signs during the interval between sample-times 2 and 3 (5 and 7 weeks of age) compared to all others (unaffected, subclinical, affected that developed clinical signs after 7 weeks of age).
[b]Excluded 10 foals that developed clinical signs before collection of sample-time 3, 7 weeks of age.

a qualitative test that simply detected the presence or absence of *vapA*. Receiver operating characteristic methods were also used to compare the fecal concentration of *vapA* in the feces of foals that developed *R. equi* pneumonia between 5 and 7 weeks of age (n = 10) and the foals that did not develop clinical signs of pneumonia at any time (n = 100). Again although the 95% confidence interval for the area under the ROC curve excluded 50%, the optimal cut-point had a poor specificity; (data not shown).

In a clinical setting, foals that developed signs of pneumonia before sampling would not be subjected to a
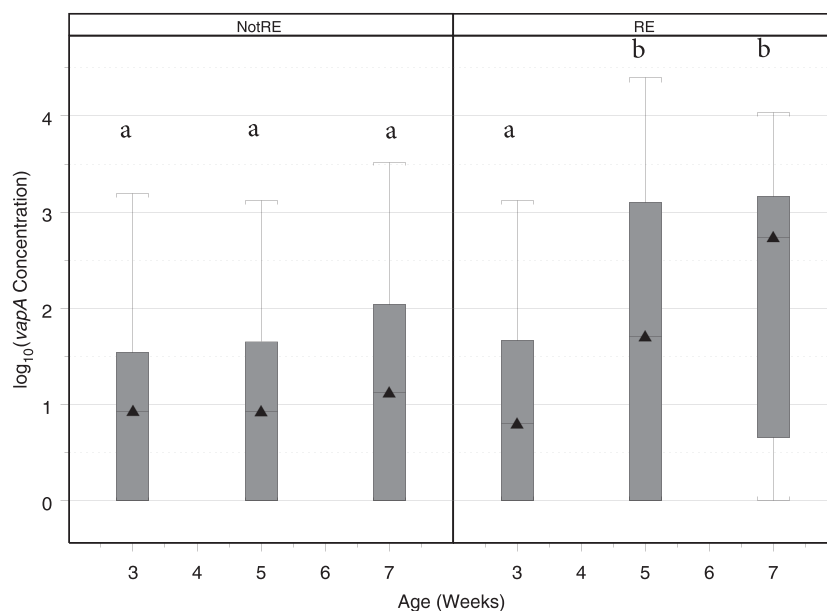
Fig 1. Boxplot of fecal concentration of *vapA* by sample-time (1, 2, or 3, corresponding to ages 3, 5, and 7 weeks, respectively), by group (NotRE = unaffected or subclinical pneumonia foals; RE = foals that developed *R. equi* pneumonia). The triangles in the boxes represent the median value; the bottom and tops of the boxes represent the 25th and 75th percentiles of the data, respectively; the thin horizontal lines from the top of the boxes extend to the 97.5th percentile. Boxes with different superscripted letters differ significantly ($P < .05$). Although the fecal concentrations of *vapA* did not increase for foals that remained free of disease (ie, subclinical and unaffected groups), fecal concentrations of *vapA* was significantly increased with age among foals that subsequently developed disease.

Pneumonia Between 5-7 Weeks

Pneumonia Between 7-9 Weeks
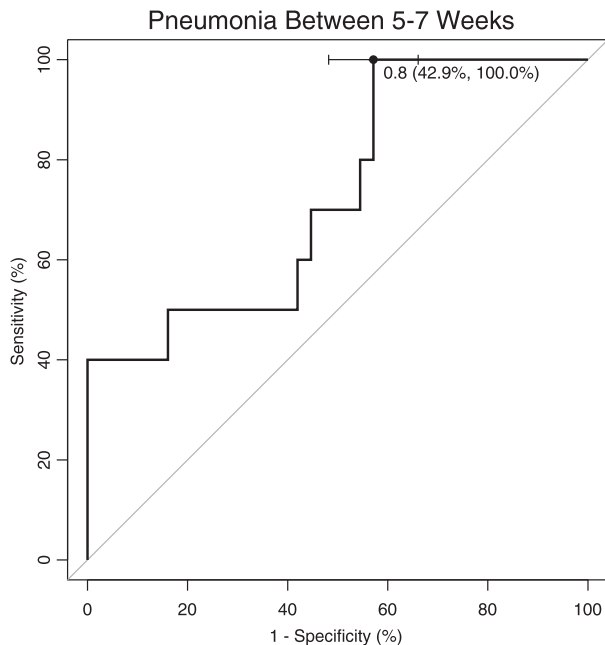
Fig 2. ROC curve for fecal *vapA* concentration at 5 weeks of age for predicting development of pneumonia during the interval between 5 and 7 weeks of age among 10 foals that developed *R. equi* pneumonia during the interval and 115 foals that did not. The thin diagonal line represents area under the ROC curve of 50% (a nondiscriminating test). The solid circle symbol represents the optimal cutoff value[15] and the value beneath it external to the parentheses is the actual copy numbers represented at this point of the curve (ie, 0.8 copies for this curve). The values inside the parentheses are the estimated specificity and sensitivity, respectively; this order is the default for the software program).
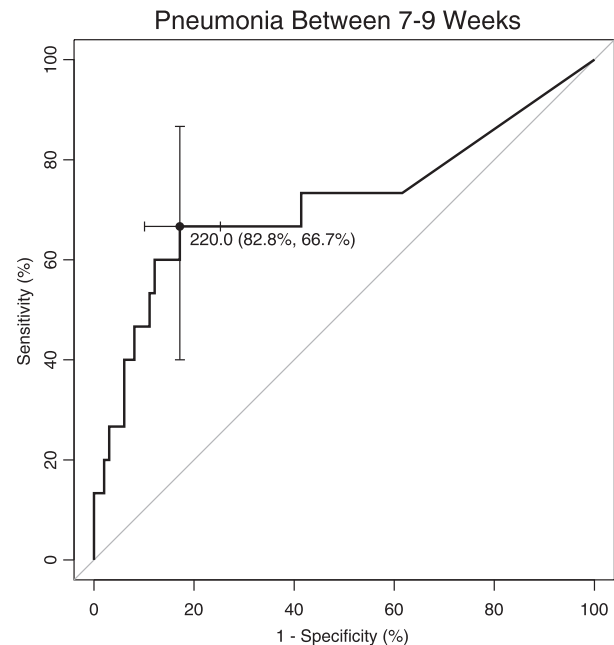
Fig 3. ROC curve for fecal *vapA* concentration at 7 weeks of age for predicting development of pneumonia after 7 weeks of age among 15 foals that developed *R. equi* pneumonia during that interval and 100 foals that did not. These data exclude the 10 foals that developed *R. equi* pneumonia between 5 and 7 weeks of age. The thin diagonal line represents area under the ROC curve of 50% (a nondiscriminating test). The solid circle symbol represents the optimal cutoff value[15] and the value beneath it external to the parentheses is the actual copy numbers represented at this point of the curve (ie, 220 copies for this curve). The values inside the parentheses are the estimated specificity and sensitivity, respectively; this order is the default for the software program).

subsequent screening test. Thus, ROC analysis for the data for 7 weeks of age was repeated excluding the 10 foals that had developed *R. equi* pneumonia between 5 and 7 weeks of age (Table 2). Although the 95% confidence interval for the area under the ROC curve excluded 50%, the area under the curve was modest (<71%) and had a weak sensitivity (67%) and moderate specificity (83%) at the optimal cut-point (Fig 3).

Discussion

The purpose of this study was to evaluate the clinical utility of serial qPCR for *vapA* in fecal samples of foals with known case outcomes from a farm with endemic *R. equi* pneumonia. A highly accurate and specific screening test would reduce the number of foals treated with macrolides on farms where the treatment is implemented on the basis of positive screening results. The importance of accurate classification of foals at screening is highlighted by the emergence of macrolide resistance in *R. equi* following mass treatment of foals with macrolides.[12]

Although there was no significant difference in fecal concentration of *vapA* (ie, virulent *R. equi*) among the multiple groups of foals (ie, affected, 3 categories of subclinical, and healthy foals) at any of the 3 sample

times, there were significant differences in concentrations between subsequently affected foals (n = 25) and all other foals (n = 100) at 5 and 7 weeks of age. The difference between groups at 5 weeks of age was more pronounced when analysis compared those foals that developed *R. equi* pneumonia during the interval from 5 weeks and 7 weeks (n = 10) either to all other foals (n = 115 foals) or to just those foals that never developed pneumonia (n = 100 foals; ie, excluding the 15 foals that developed pneumonia after 7 weeks of age). There also was a greater difference between foals that were affected after 7 weeks of age (n = 15) and foals that never developed pneumonia (n = 100) when the 10 foals that developed pneumonia before 7 weeks of age were excluded from analysis.

Results of longitudinal data analysis using mixed-effects modeling were consistent with prior findings that fecal concentrations of *R. equi* or virulent *R. equi* of foals increase with age.[19] We observed that foals that subsequently developed disease shed significantly higher concentrations of virulent *R. equi* in feces as they aged, whereas age- and birthdate-matched foals did not shed higher concentrations as they aged. These findings could indicate that affected foals and foals that remain free of disease are exposed to similar concentrations of virulent

R. equi in the first few weeks of life, and that greater exposure occurring after the first month of age results in disease. Alternatively, these findings could indicate that foals that develop pneumonia have similar exposure to virulent *R. equi* during early life to those that do not develop disease, but are more susceptible to infection and shed significantly greater numbers of virulent bacteria as pneumonia progresses. It has been demonstrated that environmental concentrations (airborne and soil) of *R. equi* are higher shortly after birth in foals that subsequently develop *R. equi* pneumonia than in foals from the same environment that remain free of disease.[20,21] Given that there were no significant differences between groups in this study at 3 weeks of age, it is possible that the fecal concentration of virulent *R. equi* is a reflection of pulmonary infection and pneumonia (where infectious pulmonary fluid moves from the lungs via the trachea to the oropharynx and is subsequently swallowed) rather than the level of exposure.

Although there were significant differences in fecal concentrations of *vapA* between the foals that subsequently became affected and all other foals at 5 and 7 weeks of age, results of ROC analysis indicated that qPCR for *vapA* on serial fecal samples was not useful as a screening test to identify foals that would develop clinical signs of pneumonia. The areas under the ROC curves indicated the overall accuracy of the test were not significantly different than 50% (an area of 50% represents a nondiscriminatory test). The area under the ROC curves for foals that would develop clinical signs of pneumonia in the subsequent 2-week interval excluded a value of 50% from the lower bound of the 95% confidence interval; nevertheless, the tests still performed inadequately for clinical utility. The estimated areas under the curve were <73%, the combination of sensitivity and specificity at the optimal cut-point for each time was 150% or less, and the cutoff value for developing pneumonia during the interval between 5 and 7 weeks was essentially equivalent to a binary present or absent outcome (ie, 0 versus >0 copies). Despite recent results from our laboratory suggesting that real-time qPCR performs with adequate accuracy for the diagnosis of *R. equi* pneumonia (among foals at the same farm as used in this study, including the foals studied here),[15] the results of this current study do not support the use of fecal qPCR for *vapA* as a screening test for *R. equi* pneumonia.

A limitation of our study is that testing was performed on fecal samples that had been stored frozen at −80°C rather than on fresh samples. Some reports describe freezing as decreasing the quality and quantity of nucleic acids in stored samples[22], whereas others show no decrease in quality or quantity of nucleic acids.[23,24] The method of cryopreservation can influence the extent of DNA degradation,[25,26] although conflicting results have been reported.[23,24] Thus, it is possible that differences between groups might have been larger and thus more discriminatory had we tested fresh fecal samples. This issue of the impact of freezing should be addressed in future studies.

Although we included an extraction control for each day that DNA extraction was performed, a limitation of our study is that we did not include either an endogenous or exogenous housekeeping gene as evidence that the DNA extraction process removed fecal inhibitors from each individual sample. Inclusion of such a housekeeping gene would have been a superior approach to the one we used. We think the impact of this limitation is likely small for the following reasons. First, the majority of our samples showed amplification. Although we cannot exclude the possibility of relative inhibition, this makes complete inhibition in samples highly improbable. Second, we were looking at relative rather than absolute differences between study groups, such that relative inhibition would have only been problematic if it were differentially distributed between cases and noncases. Last, we have used this extraction technique with success for extracting DNA both for microbiota studies and for fecal PCR.[15,27]

In summary, qPCR for *vapA* on serial fecal samples was not clinically useful as a screening test for *R. equi* pneumonia. Alternative approaches are needed to identify an accurate screening test to identify foals that will develop *R. equi* pneumonia. This study also illustrates that a significant difference in a biomarker (eg, fecal concentrations of *vapA*, serum lactate concentration, serum amyloid A concentration, etc.) measured in diseased and nondiseased animals does not necessarily indicate that the test will perform accurately to classify the current or future disease status of individual animals.

Footnotes

Acknowledgments

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