

Estimating the Sensitivity and Specificity of Real-Time Quantitative PCR of Fecal Samples for Diagnosis of *Rhodococcus equi* Pneumonia in Foals

S.D. Shaw, N.D. Cohen, M.K. Chaffin, G.P. Blodgett, M. Syndergaard, and D. Hurych

Background: Real-time, quantitative PCR (qPCR) methods for detecting *Rhodococcus equi* in feces have been developed as a noninvasive, rapid diagnostic test for *R. equi* pneumonia, but have not been evaluated in a large population of foals.

Objective: The objective of this study was to evaluate the clinical utility of fecal PCR as a diagnostic test for *R. equi* pneumonia in foals using receiver operating characteristic (ROC) methods.

Animals: 186 foals born in 2011 at an *R. equi*-endemic ranch in Texas.

Methods: Fecal samples were collected at the time of onset of clinical signs for pneumonic foals (n = 31). Foals with pneumonia were matched by age and birth date to healthy (n = 31) and subclinical (n = 124) control foals; fecal samples were collected from these controls. DNA was extracted from feces using commercial kits and concentration of virulent *R. equi* in feces was determined by qPCR.

Results: Concentration of *R. equi* in feces differed significantly ($P < .05$) among groups. The area under the ROC curve for fecal qPCR for diagnosis of *R. equi* pneumonia was 89% (95% CI, 83–99), with a sensitivity of 94% and specificity of 72%.

Conclusions and Clinical Importance: qPCR of feces can be useful as an alternative to tracheobronchial aspiration for the diagnosis of *R. equi* in foals with clinical signs of pneumonia. Caution should be used in extrapolating results of this study to other populations because fecal concentration of *R. equi* might vary by geographic location or management practices.

Key words: Coprologic diagnosis; Horse; Infectious diseases; Pneumonia; *Rhodococcus*.

Rhodococcus equi is a gram-positive, facultative, intracellular bacterium and the most important cause of severe pneumonia in foals, leading to substantial animal suffering and economic losses.¹ Both virulent and avirulent biotypes of *R. equi* have been identified.² Virulent isolates contain an 85- to 90-kb plasmid that encodes the virulence-associated protein A (VapA) that is necessary to cause disease in foals. Virulent isolates can be identified by detection of the *vapA* gene using polymerase chain reaction (PCR).^{2–8} Real-time qPCR is used for the detection of *R. equi*, because this bacterium is relatively slow growing and can be difficult to cultivate from biological samples such as feces or soil.⁹

Definitive diagnosis of *R. equi* infection in foals with signs of lower respiratory tract disease is based on bacterial culture or PCR amplification of DNA from a

Abbreviations:

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

tracheobronchial aspirate (TBA), in combination with cytologic evidence of septic pneumonia in the TBA fluid.^{1,8} The TBA procedure is invasive, labor-intensive, requires skill, carries risks to foals, and is relatively expensive. In addition, it may take up to 3 days to obtain results. For these reasons, many practitioners avoid performing a TBA in foals in a farm setting and rely on a presumptive diagnosis of *R. equi* pneumonia on the basis of signalment, clinical signs, and farm history. Thus, a noninvasive diagnostic test for *R. equi* pneumonia would be clinically valuable. To date, however, a noninvasive test has remained elusive. Serologic detection has been demonstrated to be inaccurate,^{8,10,11} and accuracy of PCR of blood samples has not been reported.

Detection of virulent *R. equi* in feces by PCR was demonstrated to be highly sensitive in a small number of foals,¹² but the need exists to further evaluate the accuracy of fecal PCR testing. Interpretation of fecal PCR results is complicated by the ecology of *R. equi*. *Rhodococcus equi* is shed in large quantities in the feces of both affected and unaffected foals, with up to 10⁶ colony-forming units (CFU) of *R. equi* per gram detected as determined by quantitative bacterial culture, gel electrophoresis, and immunoblot analysis.^{2,13–16} Thus, there is a potential for false-positive results of fecal PCR for *R. equi*.

From the Department of Large Animal Clinical Sciences, College of Biomedical Sciences and Veterinary Medicine, Texas A&M University, College Station, (Shaw, Cohen, Chaffin, Hurych); 6666 Ranch, Guthrie, TX (Blodgett, Syndergaard).

Sample collection was performed on the 6666 Ranch in Guthrie, TX. Other work was performed at the Equine Infectious Disease Laboratory in College Station, TX.

Corresponding author: N.D. Cohen, Department of Large Animal Clinical Sciences, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX 77843-4475; e-mail: ncohen@cvm.tamu.edu.

Submitted June 11, 2015; Revised July 17, 2015; Accepted August 26, 2015.

Copyright © 2015 The Authors. Journal of Veterinary Internal Medicine published by Wiley Periodicals, Inc. on behalf of the American College of Veterinary Internal Medicine.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

DOI: 10.1111/jvim.13631

The specificity of fecal testing is further complicated by the presence of foals with subclinical *R. equi* pneumonia that will not develop disease but may shed virulent *R. equi* in feces. Screening methods are used in hopes of earlier identification of foals that will develop *R. equi* pneumonia based on the rationale that earlier intervention will lead to higher success and shorter duration of treatment. Sequential thoracic ultrasonography (TUS) is a highly sensitive screening method used to detect peripheral lung abscesses.¹⁷ However, the use of TUS has indicated that many foals with findings consistent with *R. equi* infection will not develop clinical signs.^{18,a}

To the best of our knowledge, real-time qPCR of virulent *R. equi* in foal feces has not been used to differentiate between affected and unaffected foals in a population with known disease outcomes. The purpose of this study was to compare the results of qPCR for *R. equi* among foals with clinical pneumonia, subclinical pneumonia, or no evidence of clinical or subclinical pneumonia. We hypothesized that the quantity of virulent *R. equi* shed in the feces of foals at an *R. equi*-endemic farm would differ significantly among foals of the 3 groups in an ordinal manner (ie, clinical > subclinical > unaffected). We further hypothesized that a cut-point could be established for PCR testing that would be accurate for diagnosing foals with *R. equi* pneumonia.

Materials and Methods

Study Population

This study used a repository of fecal samples and existing data from a population of foals born on an *R. equi*-endemic ranch in Guthrie, Texas during 2011. All 270 foals born on the farm during 2011 that survived to weaning and that participated in a prior study^{19,20} evaluating screening tests for *R. equi* pneumonia were eligible for inclusion in this study. Thoracic ultrasonography was performed biweekly on every foal, from 3 to 16 weeks of age. The total maximal diameter (TMD) of all pulmonary consolidations was recorded for each examination. On the day of every ultrasound examination, fecal samples were collected if available, shipped chilled overnight, and stored frozen at -20°C . For this study, foals were categorized as affected, subclinical, or unaffected. Affected foals were those with clinical signs suggestive of pneumonia (eg, fever, lethargy, 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 from TBA fluid, and sonographically visible pulmonary consolidations or abscesses. Tracheobronchial aspirates were collected transendoscopically on the first day of apparent clinical signs of pneumonia and submitted for cytology and aerobic culture; *R. equi* was recovered from all affected foals. An additional fecal sample was obtained from affected foals on the day of onset of clinical signs of pneumonia. Subclinical foals 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 blinded to the results of TUS screening; no intervention was 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 at any point during the study. Forty-six (17%) foals developed *R. equi* pneumonia, 54 foals (20%) were unaffected, and 170 foals (63%) were subclinical. Protocols for this study were reviewed and approved by the Clinical Research Review Committee (CRRP 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 subject to review by the Institutional Animal Care and Use Committee.

Sample Selection

Fecal samples collected on the day of onset of clinical signs were available for 31 of the 46 affected foals. Each of these 31 affected foals was matched to an unaffected foal based on 2 criteria: age at sampling (± 7 days) and date of birth (± 30 days), to account for the effects of age and birth-month on fecal shedding. The fecal sample selected from each control foal was obtained when the foal was closest in age to its index affected foal on the day of onset of clinical signs. In the event of a tie between 2 foals regarding their selection criteria, selection priority was given to age at sampling. Each affected foal also was matched to 4 subclinical foals on the basis of age and date of birth, as described above. For each affected foal, 2 subclinical foals were selected whose lesions had a TMD ≥ 200 mm at the time of sample collection and 2 subclinical foals with a TMD < 200 mm on all ultrasonographic examinations. The rationale for including the subclinical foals with different lesion sizes was that it was considered plausible that foals with larger subclinical lesions would shed larger numbers of virulent *R. equi* in feces than would foals with smaller subclinical lesions. The cut-point of TMD = 200 mm was selected from receiver-operator characteristic curve analysis conducted for the prior screening study performed using this population.^{19,20}

Fecal DNA Extraction

Frozen fecal samples were thawed and a 1-g aliquot of feces was used for DNA extraction performed according to the manufacturer's recommendations.^a DNA also was extracted from a *vapA*⁺ reference strain (American Tissue Culture Collection [ATCC] 33701p⁺) and an isogenic plasmid-cured strain (ATCC 33701p⁻) to be used as positive and negative amplification controls, respectively. Spectrophotometry was used to quantitate total DNA. Samples were diluted with nuclease-free water to a standard concentration (20 $\mu\text{g}/\text{mL}$) and stored at -80°C until analyzed.

Standard Curve Construction

Absolute quantitation of DNA copy number required construction of a standard curve. 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), generously provided by Dr. Steve Giguère, University of Georgia. Plasmid DNA was extracted using a commercially available kit^b and the concentration was determined using spectrophotometry. Ten-fold serial dilutions of plasmid DNA (approximate range, 10^1 – 10^9 copies of pGEX-2T/ μg) were prepared in nuclease-free water. Plasmid DNA standards were processed in duplicate using real-time, qPCR. 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. A strong linear correlation ($R^2 = 0.999$; slope = -3.489) existed between the initial copy number of *vapA* and the corresponding C_T value, and the amplification efficiency was 94%.

Real-time, Quantitative PCR

Real-time, qPCR was performed as previously described.⁵ Briefly, 2 μ L of fecal DNA was added to 5 μ L of a commercial mastermix,^c 0.5 μ L of a custom premix,^d and 2.5 μ L of buffered nuclease-free water. The primer sequences used were previously designed⁵ based on the 564-bp coding sequence of *vapA* for *R. equi* strain 33701 and chosen for their specificity for *vapA*.^e Samples were processed in a real-time, qPCR unit^f using commercial software.^g Samples were tested in duplicate and measured against a standard curve of the *vapA* gene for absolute quantitation of copy numbers. Copy numbers were expressed as total copies per gram of feces. An assumption underlying our analysis was that each bacterial cell carried only 1 copy of the plasmid on which the *vapA* gene was encoded. Copy numbers of *vapA* also were calculated per g of feces based on the following formula:

$$\begin{aligned} \text{Copies of } vapA \text{ per g of feces} = & ((\text{copies of } vapA / 40 \text{ ng DNA}) \\ & \times \text{initial total DNA concentration} \\ & (\text{ng}/\mu\text{L}) \times 100\mu\text{L}) / \text{sample weight (g)}. \end{aligned}$$

where 40 ng was the total amount of DNA used per PCR reaction and 100 μ L was the total volume for each DNA extraction procedure. Assuming that each *R. equi* has 1 copy of *vapA*, the copy number per g of feces is equivalent to the number of *R. equi* per g of feces.

The intra-assay coefficients of variation (CV) determined from 10 replicates each of dilutions containing 10^4 , 10^6 , and 10^8 CFU of *R. equi* as previously described⁵ were <5%. The interassay variation determined using 3 distinct assays as previously described⁵ had a mean overall CV <2% and a mean CV across all dilutions of <1%.

Data Analysis

Analyses were performed using S-PLUS statistical software.^h For descriptive purposes, plots and summary statistics (including 95% confidence intervals) were used. Receiver-operator characteristic (ROC) curves were plotted using the pROC procedure.²¹ Comparisons of fecal copy number of *R. equi vapA* among clinical status groups (ie, affected, subclinical, and unaffected) were made using generalized linear modeling with the \log_{10} -transformed concentrations of *vapA* detected in feces as the outcome variable and study group as the dependent variable. The healthy control foals were the reference group for the linear model. The method of Sidak was used for posthoc pair-wise comparisons among groups in models with more than 2 groups. Model fit was assessed by graphical examination of diagnostic plots of residuals. A value of $P < .05$ was considered significant.

Results

The concentrations of *R. equi* in feces differed significantly among groups. The fecal *R. equi* concentrations of healthy foals and foals with lesions <200 mm did not differ significantly ($P > .05$). However, subclinical foals with lesions >200 mm had significantly ($P < .05$) higher concentrations of *R. equi* in feces than did foals in the healthy or subclinical <200 mm groups, and significantly ($P < .05$) fewer copies than age-matched foals that had *R. equi* pneumonia (Fig 1). Results were essentially identical when the data were analyzed as the number of copies per g of feces. (data not shown).

The utility of fecal PCR as a diagnostic test was assessed using ROC methods. The comparison of

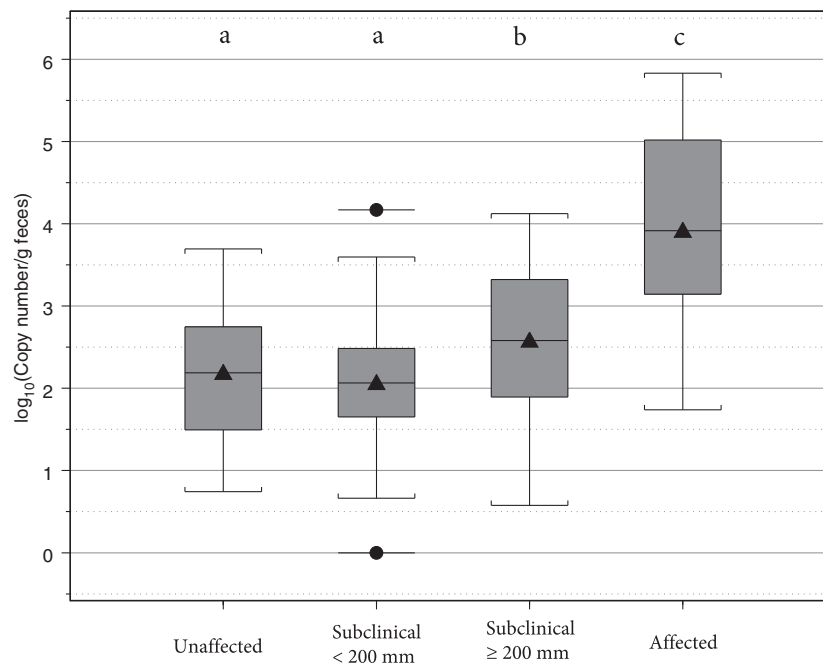


Fig 1. Boxplot of copy numbers of *vapA* per gram of feces in foals by study group. The horizontal line with the triangle is the median, and the top and the bottom of the boxed extend to the 75th and 25th percentiles, respectively. The thin vertical lines extending to thin horizontal lines represent multiples of 1.75 times their respective interquartile range, and the circles with horizontal lines represent outliers. Control – unaffected foals (n = 31); Subclinical <200 – subclinical foals with a TMD < 200 mm (n = 62); Subclinical >200 – subclinical foals with a TMD > 200 mm (n = 62); Affected – affected foals (*R. equi* pneumonia; n = 31). Different letters denote a statistically significant difference between groups ($P < .05$).

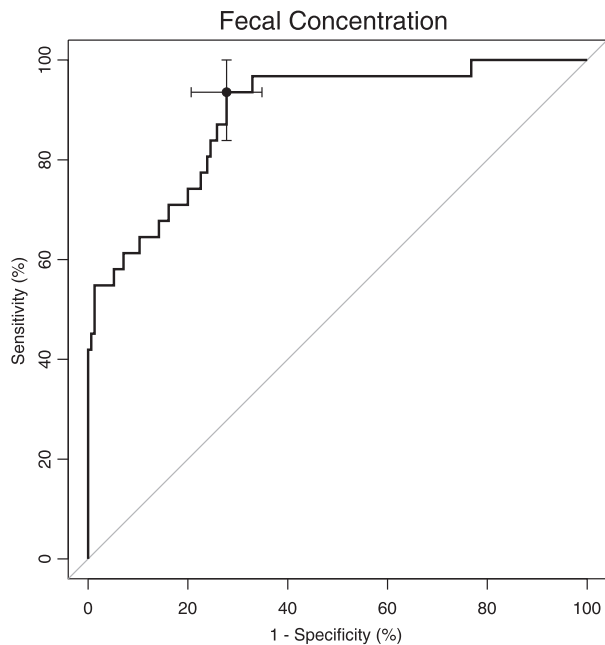


Fig 2. ROC curve (affected foals versus the other 3 groups) of the sensitivity versus (1 – specificity), both as %. The thin diagonal line represents a completely uninformative test, wherein the area under the curve (AUC) is 50%. The area under the ROC curve represented by the thicker line was 89% (95% CI, 83–99) which was significantly ($P < .05$) $>50\%$. The optimal cut-point identified from the ROC curve was $10^{2.82}$ copies (660 copies), and had a sensitivity of 94% (95% CI = 84–100) and specificity of 72% (95% CI = 65–79).

primary interest was between foals that developed *R. equi* pneumonia relative to those that did not develop pneumonia (ie, the *R. equi* group compared to the other 3 groups of foals; Fig 2). The area under the ROC curve for using fecal PCR diagnosis of *R. equi* pneumonia was 89% (95% CI, 83–99). The optimal cut-point identified from the ROC curve was $10^{2.82}$ copies (660 copies), and this cut-point had a sensitivity of 94% (95% CI, 84–100) and specificity of 72% (95% CI, 65–79).

Discussion

This study demonstrated that the use of real-time, qPCR for detection of *R. equi* in feces might be useful for the diagnosis of *R. equi* pneumonia. Fecal PCR for virulent *R. equi* is not proposed to replace the use of TBAs in clinical practice. Bacterial culture and cytologic interpretation of TBA fluid is advantageous in that one can document septic inflammation in the airways and obtain isolates for antimicrobial sensitivity testing, the latter being particularly important to guide appropriate treatment of *R. equi* pneumonia. Because TBAs are not routinely performed in the field before initiation of antimicrobial treatment, fecal PCR may help practitioners to substantiate a diagnosis for foal pneumonia and thus make more informed treatment decisions. The importance of the decision of whether or not to treat

with antimicrobials is underscored by evidence that resistance to macrolide antimicrobials is linked to mass treatment of foals.²² In the present study, qPCR had a sensitivity of 94% and specificity of 72% for the diagnosis of *R. equi* pneumonia, and an area under the ROC curve of almost 90%. These data indicate that fecal PCR could be used as a diagnostic tool yielding rapid results to direct therapeutic choices for equine practitioners. As with any diagnostic test, the positive predictive value (PPV) will vary with the pretest probability of disease. For example, a positive test result in a 2-month-old foal with clinical signs of pneumonia from a farm with an annual cumulative incidence of 20% and estimated to have a pretest probability of disease of 80% would have a PPV of 93%, whereas the PPV for a randomly selected foal from this farm (ie, pretest odds of 20%) would be only 27%.

At the time of diagnosis, foals with *R. equi* pneumonia shed significantly more virulent *R. equi* than either subclinical or unaffected foals. Furthermore, subclinical foals with larger lesions (TMD > 200 mm) shed significantly higher concentrations of virulent *R. equi* than unaffected foals and foals with smaller lesions (TMD < 200 mm), but lower concentrations than affected foals. Differences between subclinical foals with a TMD < 200 mm and those that remained unaffected throughout the course of the study were not statistically significant. Virulent *R. equi* was detected in the feces of all but 1 foal (0.5%). The high prevalence of shedding was not unexpected, because foals are exposed to virulent *R. equi* early in life²³ from a number of sources, including soil, shared air space, and the manure of dams.^{13,24,25} Clinically healthy dams shed 10^2 to $>10^3$ CFU/g.¹³ In addition, *R. equi* uses volatile fatty acids in the feces and can multiply 10^3 -fold in the environment.² These data illustrate that identification of fecal shedding of virulent *R. equi* is not synonymous with a diagnosis of disease caused by the organism.

The sensitivity and specificity of fecal PCR for the diagnosis of *R. equi* previously has been described in a study¹² that evaluated fecal shedding of virulent *R. equi* in foals with clinical signs of pneumonia. The sensitivity and specificity of fecal PCR for *R. equi* pneumonia in that study were 75% and 100%, respectively. The lower specificity in our study might be attributable to the population of subclinical foals with large pulmonary lesions. Foals with larger lesions, along with those that have clinical pneumonia, might have a relatively high concentration of virulent *R. equi* in their sputum that is being swallowed and passed through the gastrointestinal tract into their feces. Another explanation for the difference in estimated specificities is that a relatively small number of unaffected foals was used to calculate specificity in the aforementioned study and thus the precision of the estimated specificity was low.¹²

An important limitation of our study was that the population was restricted to a single, *R. equi*-endemic breeding farm. Geography, the equine population, and management conditions all have the potential to affect fecal shedding of *R. equi*. Thus, the cut-points deter-

mined in this study for the detection of *R. equi* pneumonia may not be applicable to other farms and further work is needed to determine the degree of variation in these cut-points. In addition, many of the subclinical foals in this study had large pulmonary lesions, which may not be characteristic of *R. equi* infections on other farms.

There are a number of other factors that could affect the cut-point for detection of *R. equi* pneumonia. The cut-point identified in this study may not be applicable to a similar assay developed in another laboratory because of differences in methods of DNA extraction, sample handling, standard curve preparation, and PCR reagents. Furthermore, the use of frozen feces also may have affected our cut-point. Freezing fecal samples may positively affect the extraction or stability of DNA from gram-positive bacteria.²⁶ Thus, a cut-point for diagnosis could be lower if the assay was performed on fresh feces. These considerations emphasize the need for further evaluation of this method before it can be offered commercially by diagnostic laboratories for testing.

Another important limitation was the control groups used for estimating specificity. In this study, control groups included healthy foals and foals with subclinical pneumonia. Ideally, a control group comprised of foals from the same farm with clinical signs of pneumonia and pulmonary abscesses that cultured negative for *R. equi* and positive for another causative agent, such as *Streptococcus equi* subsp. *zooepidemicus*, would have been included. Unfortunately, such foals were not found within the study population during 2011. An advantage of the controls used in this study was the inclusion of subclinical foals that were not treated with antimicrobials. The large cohort of subclinical foals was evidence of widespread exposure to *R. equi*. This finding suggests that the specificity of this fecal qPCR assay for the diagnosis of *R. equi* pneumonia was good, even in the face of subclinical disease. Although TBAs were not performed on subclinical foals to rule out other infectious organisms, polymicrobial infection has been demonstrated in up to 83% of TBAs from foals with *R. equi* pneumonia and is not significantly associated with clinical outcome.²⁷ Another limitation of our study is that we only obtained fecal samples from 31 of 46 foals (67%) with *R. equi* pneumonia. We do not believe that the absence of feces was non-random but we cannot exclude the possibility that results might have differed if we had obtained feces from all foals.

In conclusion, this study demonstrates that fecal qPCR might be useful for the diagnosis of *R. equi* pneumonia and may help guide treatment decisions when TBA samples are not collected. Fecal qPCR is a noninvasive technique with good diagnostic accuracy, making it a potential alternative to TBAs. Caution should be used in directly applying the results of this study to other populations because geographical and management factors may affect the magnitude of fecal shedding.

Footnotes

- ^a ZR Fecal DNA MiniPrep™, Zymo Research, Irvine, CA
^b Axyprep™ Plasmid MiniPrep Kit, Axygen Scientific, Union City, CA
^c TaqMan® Universal Master Mix II, Applied Biosystems, Grand Island, NY
^d Applied Biosystems, Grand Island, NY
^e VPF5, 5' AACGGTCGAGCAAGCGATAC 3' VPB6, 5' GGCCCGAATACGTGAAACCT 3'
^f Life Technologies 7900HT Fast Real-Time PCR System, Applied Biosystems, Grand Island, NY
^g SDS 2.3 software, Applied Biosystems, Grand Island, NY
^h S-PLUS statistical software, version 8.2, TIBCO, Inc., Seattle, WA
-

Acknowledgments

This work was supported by a grant from the Department of Large Animal Clinical Sciences, Texas A&M University, with additional support from the Link Equine Research Endowment. Support for sample collection was provided by a grant from the American Quarter Horse Association. This study was presented as a research abstract at the 2015 ACVIM Forum in Indianapolis, IN.

Conflict of Interest Declaration: Authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

References

- Giguère S, Cohen ND, Chaffin MK, et al. Diagnosis, treatment, control and prevention of infections caused by *Rhodococcus equi* in foals. *J Vet Intern Med* 2011;25:1209–1220.
- Dawson TRMY, Horohov DW, Meijer WG, Muscatello G. Current understanding of the equine immune response to *Rhodococcus equi*. An immunological review of *R. equi* pneumonia. *Vet Immunol Immunopathol* 2010;135:1–11.
- Monego F, Maboni F, Krewer C, et al. Molecular characterization of *Rhodococcus equi* from horse-breeding farms by means of multiplex PCR for the *vap* gene family. *Curr Microbiol* 2009;58:399–403.
- Halbert ND, Reitzel RA, Martens RJ, Cohen ND. Evaluation of a multiplex polymerase chain reaction assay for simultaneous detection of *Rhodococcus equi* and the *vapA* gene. *Am J Vet Res* 2005;66:1380–1385.
- Harrington JR, Golding MC, Martens JR, et al. Evaluation of a real-time quantitative polymerase chain reaction assay for detection and quantitation of virulent *Rhodococcus equi*. *Am J Vet Res* 2005;66:755–761.
- Arriaga JM, Cohen ND, Derr JN, et al. Detection of *Rhodococcus equi* by polymerase chain reaction using species-specific nonproprietary primers. *J Vet Diagn Invest* 2002;14:347–353.
- Rodriguez-Lazaro D, Lewis DA, Ocampo-Sosa AA, et al. Internally controlled real-time PCR method for quantitative species-specific detection and *vapA* genotyping of *Rhodococcus equi*. *Appl Environ Microbiol* 2006;72:4256–4263.
- Sellon DC, Besser TE, Vivrette SL, McConnico RS. Comparison of nucleic acid amplification, serology, and microbiologic

culture for diagnosis of *Rhodococcus equi* pneumonia in foals. *J Clin Microbiol* 2001;39:1289–1293.

9. Pusterla N, Madigan JE, Leutenegger CM. Real-time polymerase chain reaction: a novel molecular diagnostic tool for equine infectious diseases. *J Vet Intern Med* 2006;20:3–12.

10. Martens RJ, Cohen ND, Chaffin MK, et al. Evaluation of 5 serological assays to detect *R. equi* pneumonia in foals. *J Am Vet Med Assoc* 2002;221:825–833.

11. Giguère S, Hernandez J, Gaskin J, et al. Evaluation of white blood cell concentration, plasma fibrinogen concentration, and an agar gel immunodiffusion test for early identification of foals with *Rhodococcus equi* pneumonia. *J Am Vet Med Assoc* 2003;222:775–781.

12. Pusterla N, Wilson WD, Mapes S, Leutenegger CM. Diagnostic evaluation of real-time PCR in the detection of *Rhodococcus equi* in faeces and nasopharyngeal swabs from foals with pneumonia. *Vet Rec* 2007;161:272–274.

13. Grimm MB, Cohen ND, Slovis NM, et al. 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. *Am J Vet Res* 2007;68:63–71.

14. Takai S, Ohkura H, Watanabe Y, Tsubaki S. Quantitative aspects of fecal *Rhodococcus (Corynebacterium) equi* in foals. *J Clin Microbiol* 1986;23:794–796.

15. Takai S. Epidemiology of *Rhodococcus equi* infections: a review. *Vet Microbiol* 1997;56:167–176.

16. Takai S, Takahagi J, Sato Y, et al. Molecular epidemiology of virulent *Rhodococcus equi* in horses and their environments. In: Nakajima H, Plowright W, eds. *Equine Infectious Disease VII*. Newmarket: R & W Publications; 1994:183–187.

17. McCracken JL, Slovis NM. Use of thoracic ultrasound for the prevention of *Rhodococcus equi* pneumonia on endemic farms. *Proc 2005 AAEP Annu Convention* 2005;55:38–44.

18. Venner M, Rödiger A, Laemmer M, Giguère S. Failure of antimicrobial therapy to accelerate spontaneous healing of

subclinical pulmonary abscesses on a farm with endemic infections cause by *Rhodococcus equi*. *Vet J* 2012;192:293–298.

19. Chaffin MK, Cohen ND, Blodgett GP, Syndergaard M. Evaluation of ultrasonographic screening parameters for predicting subsequent onset of clinically apparent *Rhodococcus equi* pneumonia in foals. *Proc 2013 AAEP Annu Convention* 2013;59:268–269.

20. Chaffin MK, Cohen ND, Blodgett GP, Syndergaard M. Evaluation of hematologic screening methods for predicting subsequent onset of clinically apparent *Rhodococcus equi* pneumonia in foals. *Proc 2013 AAEP Annu Convention* 2013;59:267.

21. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011;12:77.

22. Burton AJ, Giguère S, Sturgill TL, et al. Macrolide- and rifampin-resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA. *Emerg Infect Dis* 2013;19:282–285.

23. Howowitz ML, Cohen ND, Takai S, et al. Application of Sartwell's model (lognormal distribution of incubation periods) to age at onset and age at death of foals with *Rhodococcus equi* pneumonia as evidence of perinatal infection. *J Vet Intern Med* 2001;15:171–175.

24. Cohen ND, Carter CN, Scot HM, et al. Association of soil concentrations of *Rhodococcus equi* and incidence of pneumonia attributable to *Rhodococcus equi* in foals on farms in central Kentucky. *Am J Vet Res* 2008;69:385–395.

25. Buntain S, Carter C, Kuskie K, et al. Frequency of *Rhodococcus equi* in feces of mares in central Kentucky. *J Equine Vet Sci* 2010;30:191–195.

26. Bahl MI, Bergstrom A, Licht TR. Freezing fecal samples prior to DNA extraction affects the *Firmicutes* to *Bacteroidetes* ratio determined by downstream quantitative PCR analysis. *FEMS Microbiol Lett* 2012;329:193–197.

27. Giguère S, Jordan LMI, Glass KG, Cohen ND. Relationship of mixed bacterial infection to prognosis in foals with pneumonia caused by *Rhodococcus equi*. *J Vet Intern Med* 2012;26:1443–1448.