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Fecal concentration of *Rhodococcus equi* determined by quantitative polymerase chain reaction of rectal swab samples to differentiate foals with pneumonia from healthy foals

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Abstract

Background: Diagnostic accuracy of real-time, quantitative PCR (qPCR) assays to quantify virulent *Rhodococcus equi* using rectal swab samples has not been systematically evaluated.

Objective: To evaluate the accuracy of qPCR of rectal swab samples to differentiate foals with pneumonia from healthy foals of similar age from the same environment.

Animals: One hundred privately owned foals born in 2021 from 2 farms in New York.

Methods: An incident case-control study design was used. Rectal swabs were collected from all foals diagnosed with *R. equi* pneumonia at 2 horse-breeding farms (n = 47). Eligible pneumonia cases (n = 39) were matched by age to up to 2 healthy (n = 53) control foals; rectal swabs were collected from control foals on the day of diagnosis of the index case. DNA was extracted from fecal swabs and the concentration of virulent *R. equi* (ie, copy numbers of the virulence-associated protein A gene [*vapA*] per 100 ng fecal DNA) was estimated by qPCR.

Results: The area under the ROC curve for qPCR of fecal swabs was 83.7% (95% CI, 74.9-92.6). At a threshold of 14 883 copies of *vapA* per 100 ng fecal DNA, specificity of the assay was 83.0% (95% CI, 71.7-92.4) and sensitivity was 79.5% (95% CI, 66.7-92.3).

Conclusions and Clinical Importance: Although fecal concentrations of virulent *R. equi* are significantly higher in pneumonic foals than healthy foals of similar age in the same environment, qPCR of rectal swabs as reported here lacks adequate diagnostic accuracy for clinical use.

KEYWORDS
foals, infectious diseases, PCR assays, pneumonia, *Rhodococcus*

Abbreviations: 95% CI, 95% confidence interval; CFU, colony forming units; IB, intrabronchial; PCR, polymerase chain reaction; pCR-VapA, plasmid into which the virulence associated protein A gene of *Rhodococcus equi* was cloned; qPCR, quantitative polymerase chain reaction; *R. equi*, *Rhodococcus equi*; ROC, receiver operating characteristic; T-TBA, trans-endoscopic tracheobronchial aspirate; VapA, virulence-associated protein A; *vapA*, gene encoding VapA.

1 | INTRODUCTION

Rhodococcus equi is gram-positive soil saprophyte and a facultative intracellular pathogen primarily recognized as a cause of foal pneumonia.[1-3] Ideally, diagnosis of *R. equi* pneumonia is based on microbiological culture and amplification of the *vapA* gene (which encodes the virulence-associated protein A [VapA] of *R. equi*) from a tracheobronchial aspirate (TBA) obtained from a foal with clinical signs of pneumonia, cytological evidence of sepsis, and radiographic or sonographic evidence of pulmonary consolidation or abscesses.[4] Collecting TBAs from foals is invasive, time- and labor-intensive, and can be unduly stressful on foals with dyspnea. Consequently, veterinarians caring for foals at large breeding farms often eschew performing TBAs and rely on findings of physical examination, clinical signs of pneumonia, hematology, and thoracic ultrasonography (TUS) to presumptively diagnose foals with *R. equi* pneumonia. The positive-predictive value of this diagnostic approach is likely high at farms with history of recurrent cases of *R. equi* pneumonia (hereafter termed endemic farms). Because of the insidious progression of *R. equi* infection to a severe pneumonia, many endemic farms have implemented the practice of using TUS to identify foals with pulmonary lesions before the onset of clinical signs and treatment of foals with lesions exceeding some threshold value for measurement of diameter of the lesion(s).[5-9] Although this process has led to reduced mortality from *R. equi* pneumonia at some endemic farms,[5-7] it also has contributed to the emergence of resistance to the antimicrobials used to treat *R. equi* in foals (viz., macrolides and rifampin).[10-17]

A noninvasive method for diagnosis of *R. equi* would be of great value to equine veterinarians to be able to more effectively identify foals that should be treated for *R. equi* pneumonia to promote better therapeutic outcomes with more judicious antimicrobial use. Although serum titers are increased in foals with *R. equi* pneumonia,[18] serological tests are not accurate.[19,20] Fecal concentrations of *R. equi* are significantly higher in pneumonic foals,[21] but quantitative microbiologic culture is time- and labor-intensive. Previously, we evaluated the accuracy of qPCR to differentiate foals with *R. equi* pneumonia from foals that remained healthy using fecal samples collected at an endemic farm in Texas.[22] Although results of that study showed good overall accuracy of the qPCR, collecting fecal samples from foals can pose logistical problems. In contrast, rectal swabs have the advantages for clinical settings of not requiring digital retrieval of feces from a foal's rectum (which might be void of fecal material) or require waiting for the foal to pass feces in a stall or other confined area. Thus, the objective of the study reported here was to evaluate the accuracy of qPCR of fecal swab samples for differentiating foals with pneumonia attributed to *R. equi* from foals that remained healthy at 2 endemic farms in New York state.

2 | MATERIALS AND METHODS

This study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

2.1 | Study cohort

The study cohort comprised foals born in 2021 and residing through weaning (at ages 150-180 days) at 2 large Thoroughbred breeding farms in the vicinity of Saratoga Springs, New York. Both farms have history of recurrent pneumonia attributed to *R. equi* using the diagnostic criteria described for foals in this study (please see below) affecting >20% of foals born at the farm during the preceding 5 years. All foals born at the 2 farms were eligible for inclusion. Our sample size was based on funding available for the project. Our goal was to include 50 foals that developed clinical signs of pneumonia attributed to *R. equi* (**cases**) and at least 50 foals that were matched to their index case for birthdate (age) by ±10 days. Because we knew that some foals selected as age-matched controls might subsequently develop pneumonia, we identified 2 control foals for each case to account for losses to ensure that we had at least 1 control for each case. Control foals were selected from the same farm as cases. Foals that were identified as controls and subsequently developed pneumonia attributed to *R. equi* were excluded from the comparisons of cases and controls; fecal swabs were collected from these foals both when they were identified as controls and when they subsequently developed pneumonia and analyzed separately.

Diagnosis of pneumonia attributed to *R. equi* was made by a veterinarian (PF-A) based on clinical signs of pneumonia, evidence of pulmonary abscesses or consolidations using TUS, and leukocytosis (WBC > 14 000 cells/μL) or hyperfibrinogenemia (>400 mg/dL). Clinical signs of pneumonia were defined as observation of ≥3 of the following findings: coughing at rest; lethargic attitude (decreased frequency of suckling, increased recumbency); rectal temperature >39.4°C; respiratory rate ≥60 breaths/min; or, increased respiratory effort. Only 5 foals (2 from farm 1 and 3 from farm 2) included in the study underwent TBA to collect fluid for microbiologic culture and cytological evaluation; in each foal, *R. equi* was isolated and cytologic evidence of sepsis was observed. For purposes of this study, control foals had none of these clinical signs; TUS was only performed in foals with any clinical signs that concerned the attending veterinarian (PF-A) or farm staff. Thus, most control foals were not examined by TUS for evidence of subclinical pneumonia.

2.2 | Fecal swabbing

Fecal swabs were collected from case foals at the time of pneumonia diagnosis and before initiating antimicrobial treatment of the index case foals and their approximate-age-matched control foals using sterile 16-in. cotton-tipped swabs (Scopettes 16″ Comfort Tip Applicators, Birchwood Laboratories, Eden Prairie, Minnesota) inserted approximately 5 cm into the rectum and gently rotated to collect fecal material from the rectal wall. Swabs were retracted and placed in sterile conical tubes, the shaft was cut, and the tubes were closed and transported to the hospital in Saratoga Springs where they were frozen at −20°C until shipped to Texas A&M University for DNA extraction.

2.3 | Real-time qPCR testing

Absolute quantification of *vapA* was performed using a standard curve, as previously described.[18,19] For the standard curve, the *vapA* gene was amplified by PCR and cloned into a plasmid vector (pCR-VapA = vector with cloned *vapA*) using a commercial kit (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Waltham, Massachusetts), transformed into an *E. coli* clone (TOP10 Cells, Invitrogen), and purified plasmid DNA was extracted using a commercial kit (QIAprep Spin Miniprep, Qiagen, Germantown, Maryland). Next, 10-fold serial dilutions of plasmid DNA (ranging from 10^1 to 10^7 copies of the pCR-VapA/2 μL) were prepared in nuclease-free water. Plasmid DNA standards were processed in triplicates on each qPCR plate. A standard curve for quantifying concentrations of *vapA* in fecal swab samples was constructed using linear regression analysis of the $\log_{10}$ quantity of *vapA* copies per sample and the corresponding C_T values, as previously described.[22,23] The linear correlation (R^2) between the initial copy number of *vapA* was determined to be >99% and the amplification efficiency estimated to be between 95% and 100% for each PCR assay.

Individual stainless steel laboratory scoops for environmental samplers (Bal Supply Scoop, Fisher Scientific, Waltham, Massachusetts) were used to remove approximately 0.1 to 0.2 mg of fecal material was removed from each frozen swab. DNA was extracted from the feces using a commercial kit (Machery-Nagel NucleoSpin DNA Stool kit, Allentown, Pennsylvania) according to the manufacturer's instructions, and resuspended to a concentration of 50 ng of fecal DNA/μL. Fecal samples were tested using real-time qPCR as previously described.[22,23] Briefly, 2 μL containing 100 ng of fecal DNA was added to 5 μL of a commercial master mix (TaqMan Fast Advanced, Applied Biosystems, Waltham, Massachusetts), 0.5 μL of a custom premix (Custom TaqMan Gene Expression Assays, Applied Biosystems), and 2.5 μL of buffered nuclease-free water. The primer sequences were designed based on the 564-bp coding sequence of *vapA* for *R. equi* strain 33 701 and for their specificity for *vapA*.[22,23] Samples were processed using a real-time, qPCR unit with commercial software (QuantStudio Real-Time PCR Software v1.3, Applied Biosystems), tested in triplicate, and measured against a standard curve of the *vapA* gene for absolute quantification of copy numbers. The median of the triplicate values was used for analysis; samples that were below the lowest standard (10 copies/100 ng fecal DNA) were assigned a value of 0 copies. It was assumed that each bacterial cell carried only 1 copy of the plasmid on which the *vapA* gene was encoded.

2.4 | Data analysis

Analyses were performed using R statistical software (Version 4.1.2, R Core Team, Vienna, Austria). For descriptive purposes, plots and summary statistics (including 95% confidence intervals [95% CIs]) were used. Receiver-operator characteristic (ROC) curves were plotted using the pROC package in R.[20] The optimal cut-point for diagnosis was selected using the best thresholds option.[24] Comparisons of fecal copy number of *R. equi vapA* copies between pneumonia cases and healthy controls by farm were made using generalized linear modeling (glm) using R. Copy numbers were $\log_{10}$-transformed for analysis to conform with distributional assumptions underlying the glm. The $\log_{10}$-transformed concentrations of *vapA* detected in feces were the dependent (outcome) variable and study group and farm were modeled as independent variables. The method of Tukey was used for post hoc pair-wise comparisons between groups using the multcomp package in R. Model fit was assessed by graphical examination of diagnostic plots of residuals. The fecal concentrations of *vapA* for the 8 foals that were initially identified as controls but subsequently developed pneumonia (and that were excluded from ROC and glm analysis) were analyzed. The data were summarized using medians and ranges and the paired differences compared using a Wilcoxon sign-rank test because the data from foals when they were determined to be cases were not deemed to be normal on the basis of a Shapiro-Wilks test ($P < .001$ that the data were derived from a normal distribution). A value of $P < .05$ was considered significant for all analyses.

3 | RESULTS

During 2021, 98 foals were born at farm 1, of which 68 remained at the farm through weaning and were eligible for inclusion in the study. Of those 68 foals, 15 (22%) developed pneumonia attributed to *R. equi*. During 2021, 126 foals were born at farm 2, of which 90 remained at the farm through weaning and were eligible for inclusion in the study. Of those 90 foals, 37 (41%) developed pneumonia attributed to *R. equi*. A total of 39 cases and 53 healthy controls were included in the ROC and glm. Nineteen foals (9 pneumonia cases and 10 controls) were from farm 1 and 73 foals were from farm 2 (30 pneumonia cases and 43 controls). No foals at either farm were diagnosed with pneumonia caused by agents other than *R. equi*, and no foals at either farm were identified with fewer than 3 of the specified clinical signs. Eight foals (1 from farm 1 and 7 from farm 2) that were initially identified as controls matched to pneumonia cases were excluded from ROC and glm analysis; changes in fecal *vapA* concentrations between when they were identified as controls and subsequently developed pneumonia were evaluated for these 8 foals.

The area under the ROC curve for using fecal PCR to differentiate cases of pneumonia attributed to *R. equi* from healthy controls was 83.7% (95% CI, 74.9-92.6). The optimal cut-point identified from the ROC curve was 14 883 copies of vapA per 100 ng fecal DNA (Figure 1). At this cut-point, specificity of the assay was 83.0% (95% CI, 71.7-92.4) and sensitivity was 79.5% (95% CI, 66.7-92.3).

The fecal concentrations of *vapA* in the feces of control foals did not differ significantly ($P = .7560$) for farm 1 (mean, 3757 copies/100 ng DNA; 95% CI, 1044-13 516 copies/100 ng DNA) and farm 2 (mean, 1854 copies/100 ng DNA; 95% CI, 448-7677 copies/100 ng/DNA). Fecal *vapA* concentrations in feces of pneumonia cases at farm 1 (mean, 210 426 copies/100 ng DNA; 95% CI,

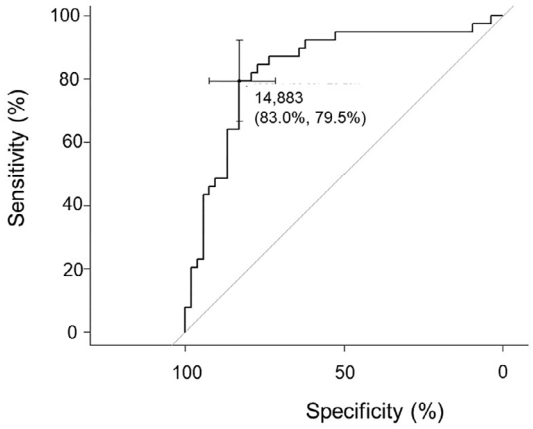

FIGURE 1 ROC curve of the sensitivity versus specificity (both as %) for concentration of virulent *R. equi* in feces (*vapA* copies/100 ng fecal DNA). The thin diagonal line represents a completely uninformative test for which the area under the ROC curve (AUC) is 50%. The area under the ROC curve presented by the thicker line was 83.7% (95% CI, 74.9-92.6) which was significantly ($P < .05$) >50%. The optimal cut-point identified from the ROC curve was 14883.4 *vapA* copies/100 ng DNA, and had a sensitivity of 83.0% (95% CI, 71.7-92.4) and specificity of 79.5% (95% CI, 66.7-92.3)

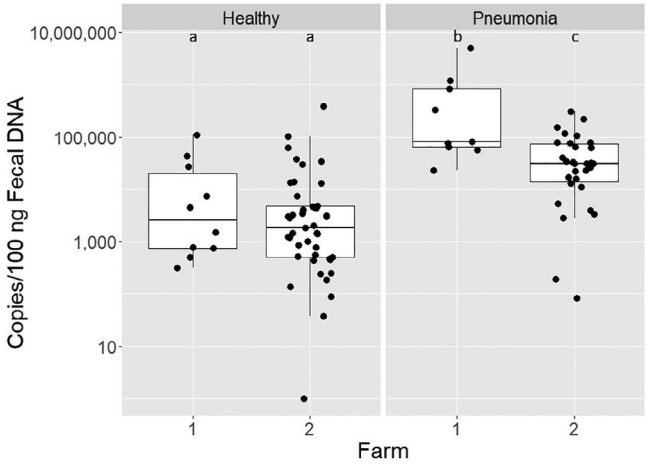

FIGURE 2 Boxplots of copies of *vapA* gene of virulent *R. equi* per 100 ng DNA from feces of 92 foals from 2 farms in New York faceted by disease status (left panel, healthy controls; right, panel, cases of pneumonia attributed to *R. equi*). The top and bottom of each box are the 75th and 25th percentiles of the data, respectively; the horizontal line bisecting the box is the median (50th percentile). Thin line (whiskers) extending above and below each box are a multiple (viz., 1.75) of the interquartile distance. Dots represent the observed values from foals. Groups with different letters above their boxplot differ significantly ($P < .05$) after adjusting for multiple comparisons. At both farms, concentrations of virulent *R. equi* from fecal swab samples were significantly greater in cases of pneumonia attributed to *R. equi* than controls, and the magnitude of difference between cases and controls was nearly 2 logs. Concentrations of virulent *R. equi* were significantly lower for foals with pneumonia attributed to *R. equi* at farm 1 than farm 2, but concentrations of healthy controls did not differ significantly between farms

32 763-1 351 500 copies/100 ng DNA) were significantly ($P < .001$) greater than those for controls from farm 1 (Figure 2). Similarly, fecal *vapA* concentrations in feces of pneumonia cases at farm 2 (mean, 21 426 copies/100 ng DNA; 95% CI, 4984 to 95 808 copies/100 ng DNA) were significantly ($P < .001$) greater than those for controls from farm 2 (Figure 2). Moreover, fecal concentrations for pneumonia cases from farm 1 were significantly ($P = .019$) higher than those for farm 2.

For the 8 foals that were initially identified as controls but subsequently developed pneumonia attributed to *R. equi*, the median fecal *vapA* concentration for the control sample was 14 330 copies/100 ng DNA (range, 215-72 293 copies/100 ng DNA), and the median fecal *vapA* concentration when the foals were pneumonia cases was 216 948 copies/100 ng DNA (range, 4366- 1 729 308 copies/100 ng DNA). The median difference between samples was 170 684 copies/100 ng DNA (range, −37 465 to 1 724 913). The paired differences in *vapA* concentrations between when foals were pneumonia cases minus controls was not significant ($P = .055$). Of the 8 foals, fecal swab *vapA* concentrations were higher when they were pneumonia cases than when identified as controls for 7 foals. The median days between case and control samples for these 8 foals was 13 days (range, 6-24 days).

4 | DISCUSSION

An accurate minimally invasive test for diagnosis of foals presumed to have pneumonia caused by *R. equi* would be useful for equine veterinary medicine because TBAs are not routinely performed by veterinarians at farms where *R. equi* is endemic and because overuse of antimicrobials to treat foals with subclinical pneumonia has contributed to the emergence of an alarming level of antimicrobial resistance in *R. equi* isolated from foals and their environment in the United States.[10-17] For this reason, we investigated the ability of qPCR of fecal swabs (which can be collected conveniently from foals) to differentiate between cases of pneumonia attributed to *R. equi* and healthy controls at 2 horse breeding farms in New York. Fecal concentrations of *vapA* from rectal swab samples were significantly greater in samples from foals with pneumonia attributed to *R. equi* than age-matched controls and the typical magnitude of difference was nearly 100-fold (Figure 2). Despite these findings, ROC curve analysis revealed that the area under the ROC curve was 83.7% (95% CI, 74.9-92.6), and at the optimal cut-point for diagnosis specificity of the assay was 83.0% (95% CI, 71.7-92.4) and sensitivity was 79.5% (95% CI, 66.7-92.3). Although the area under the ROC curve was relatively large and significantly better than 50% (the area under the ROC curve for a useless test), these results should not be considered strong evidence favoring use of the assay as described here for diagnostic purposes. Case-control designs are not optimal for assessing diagnostic tests because they often consider extremes of phenotypes, viz. florid cases of disease versus healthy controls, resulting in exaggerated estimates of diagnostic accuracy.[25-27] Thus, we would expect the qPCR

test as described here to perform less accurately as described in this case-control study than it would in a well-designed study to differentiate between foals with clinical signs of pneumonia attributed to *R. equi* and foals that have signs of pneumonia attributable to another cause (eg, streptococcal foal pneumonia).

Although concentrations of *vapA* in feces of rectal swabs from healthy foals did not differ between farms, fecal concentrations differed significantly between farms among foals that had pneumonia. This underscores the importance of evaluating the test using unbiased sampling of foals from multiple farms to determine the extent to which farm-to-farm variation might impact the accuracy of using qPCR of fecal swabs for diagnosis of pneumonia attributed to *R. equi*.

We standardized the amount of fecal DNA tested for each sample. Nevertheless, opportunities exist to modify the methods used in this study to improve assay accuracy. Samples were collected and stored frozen for varying periods before shipping from New York to Texas on a bi-weekly basis. No transport medium was used for swab samples, and no standards were established for the quantity of fecal matter on the rectal swab samples. It is conceivable that test performance could have been improved by using fresh samples submitted in a selective culture medium with a standardized amount of fecal material. Alternatively, using freshly voided fecal samples to yield a larger specimen that could be homogenized and sub-sampled before testing might improve accuracy.

We excluded all foals that were identified as controls that subsequently developed pneumonia attributed to *R. equi* from our ROC and generalized linear modeling analysis and examined the data from these 8 foals separately. Five of these 8 foals (62.5%) had concentrations above the threshold for diagnosis (14 833 copies of *vapA*/100 ng/DNA). Surprisingly, 1 foal that had a high concentration of *vapA* in its control sample (41 831 copies of *vapA*/100 ng/DNA) had a concentration below the selected cut-point when it was diagnosed with pneumonia attributed to *R. equi* (4366 copies of *vapA*/100 ng/DNA). We are unsure of the reasons for this discrepant finding. The triplicate values from the qPCR for this sample were similar, and we did not find evidence of mislabeling or mishandling of this specimen in the laboratory. The foal was not treated with antimicrobials before collecting the case sample. This lower fecal concentration of *vapA* at the time of diagnosis resulted in a nonsignificant difference for the Wilcoxon sign-rank test comparing the paired differences between pneumonia case and control samples for these 8 foals.

This study had important limitations. First and foremost, a case-control study design is not optimal for assessing accuracy of diagnostic testing.[25-27] But case-control studies are a useful starting point for evaluating diagnostic tests: in general, a test that is not highly accurate for distinguishing between cases and controls is not suitable for further evaluation for diagnostic testing. The standard for diagnosis of *R. equi* pneumonia was not used to identify cases and to establish relative diagnostic accuracy. It will be difficult to conduct a study using this reference standard because TBAs are rarely performed in the field and we are not aware of any large horse breeding farms that perform TBAs for all foals with pneumonia or that would be willing to consent to such testing. Our diagnostic criteria for pneumonia attributed to *R. equi* likely have a high positive predictive value because both farms have history of recurrent cases of foals with *R. equi* pneumonia, including cases confirmed by microbiologic culture, other causes of pneumonia abscessing pneumonia (such as *Streptococcus equi*) were not identified at either farm during the year of the study or in the preceding 5 years, and microbiological culture and cytology findings confirmed the diagnosis of *R. equi* pneumonia in the 5 foals that underwent this testing. We examined a relatively small group of foals at only 2 farms. Our 2 farms were limited to a single geographic region in the United States and cannot be generalized to other farms or other areas. Our finding of a significant difference between the 2 farms in the fecal concentrations of *vapA* among foals with pneumonia points to the need to evaluate fecal concentrations of *vapA* among pneumonic foals at multiple farms. Although it is generally assumed that each *R. equi* harbors a single copy of the large plasmid encoding VapA (approximately 85 kb), we did not attempt to determine the validity of this assumption. Nevertheless, it is unlikely that the number of plasmid copies could explain the large magnitude of difference in copy numbers between cases and controls.

In summary, although rectal swabbing is a convenient method for sampling foals for qPCR to estimate the concentration of virulent *R. equi* in feces and cases of pneumonia attributed to *R. equi* had significantly higher fecal concentrations of virulent *R. equi* than healthy foals from the same environment, results of this study indicate further optimization of the method reported here is needed before further evaluation of the accuracy of qPCR of copy numbers of *vapA* from rectal swab samples of foals is merited. Evaluation of diagnostic accuracy should include more rigorous study design to include sequential sampling of samples collected from foals at multiple farms with predetermined definitions for eligibility, inclusion, and exclusion, blinding and independence of the testing laboratory/laboratories, and—if possible—simultaneous use of a reference standard (ie, microbiological culture [with or without PCR of isolates of *R. equi* for the *vapA* gene] and cytological examination of fluid obtained by tracheobronchial aspiration) from all foals or a representative sample of included foals. This study also demonstrates that significant differences in a test result between groups does not ensure that the test result will perform accurately for classifying the disease status or prognosis of individual animals.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

ETHICS STATEMENT

This study was approved by the Texas A&M University IACUC and the Clinical Research Review Committee of the Texas A&M College of Veterinary Medicine & Biomedical Sciences (Protocol 2020-0280).

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