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Association of pneumonia with concentrations of virulent *Rhodococcus equi* in fecal swabs of foals before and after intrabronchial infection with virulent *R. equi*

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Abstract

Background: Intragastric administration of virulent *Rhodococcus equi* protects foals against subsequent experimental intrabronchial (IB) infection, but it is unknown whether *R. equi* naturally ingested by foals contributes to their susceptibility to pneumonia.

Hypothesis: Fecal concentration of virulent *R. equi* before IB infection with *R. equi* is positively associated with protection from pneumonia in foals.

Animals: Twenty-one university-owned foals.

Methods: Samples were collected from experimental studies. Five foals were gavaged with live, virulent *R. equi* (LVRE) at age 2 and 4 days; the remaining 16 foals were not gavaged with LVRE (controls). Fecal swabs were collected from foals at ages 28 days, immediately before IB infection. Foals were monitored for clinical signs of pneumonia, and fecal swabs were collected approximately 2 weeks after IB infection. Swabs were tested by quantitative PCR for concentration of virulent *R. equi* (ie, copy numbers of the virulence-associated protein A gene [*vapA*] per 100 ng fecal DNA).

Results: Fecal concentrations of virulent *R. equi* (*vapA*) before IB infection were significantly ($P < .05$) lower in control foals (25 copies/100 ng DNA [95% CI, 5 to 118 copies/100 ng DNA) that developed pneumonia (n = 8) than in healthy control foals (n = 8; 280 copies/100 ng DNA; 95% CI, 30 to 2552 copies/100 ng DNA) or those gavaged with LVRE (707 copies/100 ng DNA, 95% CI, 54 to 9207 copies/100 ng DNA).

Conclusions and Clinical Importance: Greater natural ingestion of LVRE might contribute to protection against pneumonia among foals.

Abbreviations: 95% CI, 95% confidence interval; CFU, colony forming units; IB, intrabronchial; LVRE, live, virulent *Rhodococcus equi*; 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*; T-TBA, transendoscopic tracheobronchial aspirate; VapA, virulence-associated protein A.

KEYWORDS
equid, neonatology, PCR assays, pneumonia, *Rhodococcus*

1 | INTRODUCTION

Rhodococcus equi is gram-positive, soil saprophyte and a facultative intracellular pathogen primarily recognized as a cause of foal pneumonia.[1-3] Strains that are virulent in foals carry a plasmid that encodes the virulence-associated protein A (VapA).[3,4] VapA-positive strains are virtually ubiquitous in the environment of foals, including soil, air, and the feces of their dams.[2,5-14] The ubiquity of *R. equi* in horses and their environment complicates efforts to investigate immune responses to experimental infection with or immunization against this bacterium because of the ineluctable confounding effects of natural exposure. Moreover, the impact of natural exposure to *R. equi* on immunity to this bacterium appears complex. The airborne burden of virulent *R. equi* has been associated with increased odds of disease at the levels of farm[7] and individual foal,[10,11] suggesting that greater airborne exposure increases the risk of *R. equi* pneumonia in foals, presumably through inhalation of virulent isolates. In contrast, epidemiological investigations have demonstrated that concentrations of virulent *R. equi* in soil from horse farms or in mare feces are not associated with increased odds of pneumonia in foals.[7,8,12] However, intragastric administration of virulent *R. equi* to foals provides protection against subsequent intrabronchial (IB) infection.[15,16] This suggests that coprophagia or ingestion of soil by foals might provide some degree of protection through natural exposure. This hypothesis is further supported by the evidence of varying incidence of pneumonia among foals in the same environment at farms.[2,5,7,8,12,17-19] Varying enteral exposure to ingested or swallowed *R. equi* might contribute to the varying incidence of disease observed in foals with similar natural or experimental exposure to *R. equi*.

The concentration of virulent *R. equi* in feces cannot be determined directly by quantitative culture because it is necessary to differentiate virulent from avirulent strains using either immunoassays to detect VapA protein (such as immunoblotting)[13,14] or nucleic-acid based methods (such as polymerase chain reaction [PCR]) to detect *vapA*.[20-22] Our laboratory has developed a quantitative PCR (qPCR) for detection of absolute copy numbers of *vapA* in fecal samples based on a standard curve and the assumption that each *R. equi* has a single copy of *vapA*.[21,22] Fecal qPCR can be used to quantify the concentration of virulent *R. equi* in feces by testing a standardized amount of either fecal mass or fecal DNA. Foals with *R. equi* pneumonia have been demonstrated to shed higher concentrations of virulent *R. equi* than healthy foals of similar age.[21,23-27]

We have noted variability in the proportion of foals that develop clinical signs of pneumonia to IB infection of the same dose (eg, 1×10^6 colony forming units [CFU]) of the same strain of virulent *R. equi* administered to foals in the same environment experiencing similar management (unpublished data in addition to cited references).[28-30] Given that intragastric administration of virulent *R. equi* protects foals against subsequent IB challenge,[15,16] we hypothesized that foals that developed clinical signs of pneumonia after IB infection with virulent *R. equi* would have lower fecal concentrations of *R. equi* as determined by qPCR before IB infection than foals that were infected with the same strain at the same age that did not develop pneumonia. We further hypothesized that foals that developed clinical signs of pneumonia would have significantly higher concentrations of virulent *R. equi* in feces at the time of onset of clinical signs than foals of the same age that did not develop pneumonia after infection.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

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

2.2 | Study cohort

The study cohort was a convenience sample of 21 foals born in 2021 being used in research projects addressing other specific aims. Thus, no a priori sample size calculations were performed. All foals were Quarter Horse foals that were healthy at birth, had transfer of passive immunity assessed by a semiquantitative immunoassay (SNAP Foal IgG Test, IDEXX, Westbrook, ME, USA), and results of complete blood counts (CBCs) within reference ranges at ages 1 and 28 days. Five foals were gavaged at ages 2 and 4 days with approximately 2×10^{10} colony forming units (CFU) live, virulent *R. equi* (LVRE) using strain ATCC reference strain 33701 (ATCC, Manassas, Virginia); bacteria were suspended in 120 mL sterile saline for each treatment and were administered via nasogastric intubation. These 5 foals were part of a project studying immune responses to intragastric administration of LVRE (gavaged foals). The remaining foals ($n = 16$) were used in a study of immunity to IB infection with virulent *R. equi* (control foals). Foals were maintained in stalls with their dams for 7 days after foaling, then housed in paddocks with 2 or 3 other mare-foal pairs until age 28 days. All foals were housed and maintained in paddocks at a single facility.

2.3 | Experimental infection

Before IB infection with *R. equi*, each foal's lungs were auscultated and examined by thoracic ultrasonography to document absence of preexisting pulmonary disease. Ultrasound examinations were repeated weekly after infection to monitor for lung lesions. At age

28 days, foals were sedated with xylazine (0.2 mg/kg) and butorphanol tartrate (0.04 mg/kg) and then infected transendoscopically with 25 mL of *R. equi* suspension containing 1×10^6 CFU of a strain derived from a pneumonic foal infused into each mainstem bronchus (right and left).[28,29] Approximately 200 μL of the challenge dose was saved to confirm the concentration (dose) administered, and to verify virulence of the isolate using multiplex PCR.

2.4 | Fecal swabbing

Fecal swabs were collected from 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 laboratory where they were frozen at −80°C until DNA extraction. Fecal swabs were collected from each foal twice. The first sample was collected from all foals immediately before IB infection at age 28 days. Foals that developed pneumonia were sampled at the onset of clinical signs of disease (median, 14 days after infection; range, 12-19 days after infection) or at age 42 days (14 days after infection) from foals that remained healthy.

2.5 | Clinical monitoring

Foals were observed twice daily from birth for signs of disease by the authors. The person determining pneumonia status (NDC) was masked to the treatment status of the foals. Beginning at age 28 days (the day of infection), rectal temperature, heart rate, respiratory rate, signs of increased respiratory effort (abdominal lift, flaring nostrils), presence of abnormal lung sounds (crackles or wheezes, evaluated for both hemithoraces), presence of abnormal tracheal sounds, coughing, signs of depressed attitude (subjective evidence of increased recumbence, lethargy, reluctance to rise), and nasal discharges were monitored, and results recorded twice daily through age 84 days. Thoracic ultrasonography was performed on the day of infection (before infection) and then weekly to identify evidence of peripheral pulmonary consolidation or abscess formation consistent with *R. equi* pneumonia. Foals were considered to have clinical signs of pneumonia if they demonstrated ≥3 of the following clinical signs: coughing at rest; depressed attitude; rectal temperature >39.4°C; respiratory rate ≥60 breaths/min; or, increased respiratory effort. Foals were diagnosed with *R. equi* pneumonia if they had clinical signs of pneumonia, ultrasonographic evidence of pulmonary abscess formation or consolidation with a maximal diameter of ≥2.0 cm, positive culture of *R. equi* from transendoscopic tracheobronchial aspirate (T-TBA) fluid, and cytologic evidence of septic pneumonia from T-TBA fluid. Foals diagnosed with *R. equi* pneumonia were treated with a combination of clarithromycin (7.5 mg/kg; PO; q 12 hours) and rifampin (5 mg/kg; PO; q 12 hours) until both clinical signs and thoracic ultrasonography lesions had resolved. Foals also were treated as deemed necessary by attending veterinarians with flunixin meglumine (0.6 to 1.1 mg/kg; PO; q 12 to 24 hours) for inflammation and fever.

2.6 | Real-time qPCR testing

Absolute quantification was performed using a standard curve based on the *vapA* gene, as previously described.[21,22] 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 *Escherichia coli* clone (TOP10 Cells, Invitrogen), and purified plasmid DNA was extracted using a commercial kit (QIAprep Spin Miniprep, Qiagen, Germantown, Maryland). Ten-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 was constructed using linear regression analysis of the $\log_{10}$ quantity of *vapA* copies per sample and the corresponding C_T values. For all PCR runs, 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%, as previously described.[21,22]

Fecal material was removed from frozen swabs using stainless steel laboratory scoops for environmental samplers (Bal Supply Scoop, Fisher Scientific, Waltham, Massachusetts). An individual scoop was used for each sample to remove between 0.1 and 0.2 mg of fecal material from each swab. Fecal DNA was extracted using a commercial kit (Machery-Nagel NucleoSpin DNA Stool kit, Allentown, Pennsylvania) per the manufacturer's instructions, and resuspended to a concentration of 50 ng of fecal DNA/μL. Real-time, qPCR was performed as previously described.[21,22] Briefly, 2 μL (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 previously designed[21,22] based on the 564-bp coding sequence of *vapA* for *R. equi* strain 33701 and chosen for their specificity for *vapA*. Samples were processed in a real-time, qPCR unit using commercial software (QuantStudio Real-Time PCR Software v1.3, Applied Biosystems). Samples were 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. An assumption underlying our analysis was that each bacterial cell carried only 1 copy of the plasmid on which the *vapA* gene was encoded.

2.7 | Data analysis

The primary aim of the study was to compare the fecal concentrations (copies of *vapA*/100 ng fecal DNA) of virulent *R. equi* before and after

IB infection among 3 groups of foals (control foals that developed pneumonia, control foals that did not develop pneumonia, and gavaged foals [that did not develop pneumonia]). Data were analyzed using descriptive and inferential methods. For descriptive purposes, categorical data were summarized as proportions and continuous data were summarized as either means and standard deviations (SDs) or medians and ranges when data distribution appeared non-Gaussian. For inferential analysis, we compared the fecal concentrations of *vapA* between groups and sample-times (ie, before and approximately 2 weeks after infection) using a linear mixed-effects model with an exchangeable correlation matrix, with study group and sample-time modeled as fixed, categorical effects and foal modeled as a random effect; analysis was performed using the lme function in the nlme package of R statistical software (Version 4.0.4, R Core Team, Vienna, Austria). Post hoc multiple comparisons between treatment groups and sampling times were made with the method of Tukey using the multicomp package in R statistical software. Copy numbers were $\log_{10}$-transformed for analysis to conform with distributional assumptions underlying the linear mixed-effects modeling. Maximum-likelihood methods were used to estimate 95% confidence intervals (95% CIs) of model estimates. Model-based estimates were back transformed (ie, exponentiated to power of 10). The birth-month was compared among groups of foals using Fisher's exact test using R, and the fecal concentration of virulent *R. equi* was compared between a binary variable for birth-month using a Wilcoxon rank-sum test using R. For all analysis, a significance level of $P < .05$ was used.

3 | RESULTS

Consistent with previous reports,[15,16] none of the foals gavaged with LVRE at ages 2 and 4 days developed pneumonia. Of the 16 control foals (not gavaged with LVRE), 8 (50%) developed clinical signs of pneumonia. All pneumonic foals were treated successfully and recovered without any residual signs or sequelae.

The concentrations of virulent *R. equi* in feces differed significantly among groups in manner that depended on sample time (Figure 1). Before infection, the estimated fecal concentrations of virulent *R. equi* in feces of control foals that subsequently developed pneumonia (mean, 25 copies/100 ng DNA; 95% CI, 5 to 118 copies/100 ng DNA) were significantly ($P = .04$ and $P = .01$, respectively) lower than those for control foals that were healthy (mean, 280 copies/100 ng DNA; 95% CI, 30 to 2552 copies/100 ng DNA) and those that were gavaged with LVRE and remained healthy (mean, 707 copies/100 ng DNA; 95% CI, 54 to 9207 copies/100 ng DNA). The difference in fecal concentrations of virulent *R. equi* between the healthy control group and the gavaged group before IB infection was not significant ($P = .97$).

The onset of pneumonia occurred between 12 and 19 days after infection (median, 14 days) among the 8 control foals that developed clinical signs. Fecal concentrations of virulent *R. equi* increased significantly ($P < .001$) among the pneumonic control foals from a mean of 25 copies/100 ng DNA before IB infection (95% CI, 5 to 118 copies/100 ng DNA) to a mean of 9401 copies/100 ng DNA (95% CI, 1217 to 72 646 copies/100 ng DNA) after IB infection, nearly a 400-fold increase. The fecal concentration of virulent *R. equi* rose after IB infection in healthy control foals from a mean of 280 copies/100 ng DNA (95% CI, 29 to 2552 copies/100 ng DNA) before IB infection to a mean of 4116 copies/100 ng DNA (95% CI, 433 to 39 102) at age 42 days (2 weeks after IB challenge)—a nearly 15-fold increase—but this difference was not significant ($P = .06$) after adjusting for multiple comparisons. In the gavaged foals, fecal concentrations of virulent *R. equi* decreased significantly ($P < .001$) from a mean of 707 copies/100 ng DNA (95% CI, 54 to 9207 copies/100 ng DNA) before IB infection to 4 copies/100 ng DNA (95% CI, < 1 to 45 copies/100 ng DNA) after IB infection. The concentrations of virulent *R. equi* in feces after IB infection of foals that were gavaged were significantly ($P < .001$) lower than those of either the healthy control foals or the pneumonic control foals; however, no significant difference ($P = .97$) was observed in the fecal concentrations after IB infection between the healthy control foals and the control foals that developed pneumonia.

Foals were born in 2021 between the months of January and May (Table 1), and the distribution of birth-month differed

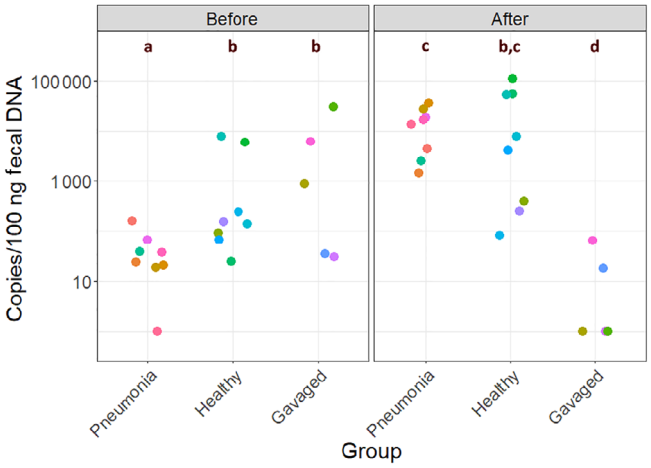

FIGURE 1 Copies of *vapA* gene of virulent *R. equi* per 100 ng DNA from feces of 21 foals in 3 groups of foals infected intrabronchially (IB) with virulent *R. equi* at age 28 days: (1) control foals that developed pneumonia after IB infection (Pneumonia; n = 8); (2) control foals that remained healthy after IB infection (Healthy; n = 8); and (3) foals that were gavaged with live, virulent *R. equi* at ages 2 and 4 days (Gavaged; n = 5). The left panel (before) represents concentrations on the day of IB infection (age 28 days, before IB infection) and the right panel (after) represents samples collected after IB infection with *R. equi* (age 42 days for healthy and gavaged foals and collected between 12 and 19 days [median, 14 days]) for pneumonia foals). Groups with different maroon letters differ significantly ($P < .05$) after adjusting for multiple comparisons. Colors of dots represent different foals within treatment group. Concentrations of virulent *R. equi* were significantly lower before IB infection for foals in the pneumonia foal relative to the other 2 groups. Concentrations of virulent *R. equi* increased significantly after challenge for control foals that developed pneumonia but decreased significantly among foals in the gavaged group

TABLE 1 Birth-month of foals infected intrabronchially with virulent *R. equi* at age 28 days by study group: (1) control foals that remained healthy (Healthy); (2) control foals that developed pneumonia (Pneumonia); and (3) foals that were gavaged (Gavaged) with live, virulent *R. equi* at ages 2 and 4 days

Month(s)	Healthy	Pneumonia	Gavaged
January	0	1	0
February	0	0	1
March	0	6	1
April	3	1	2
May	5	0	1
January to March	0	7	2
April or May	8	1	3

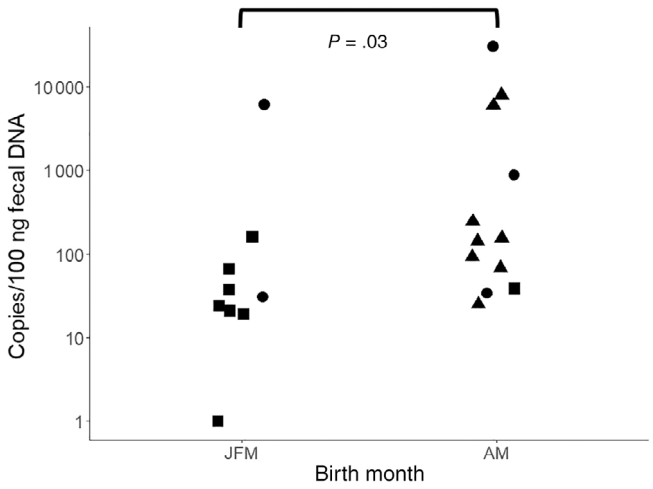

FIGURE 2 Copies of *vapA* gene of virulent *R. equi* per 100 ng of fecal DNA from 21 foals at age 28 days (before intrabronchial [IB] infection) by birth-month: JFM = January, February, or March; and AM = April or May. Dots are colored by study group: (1) triangles represent control foals that remained healthy after IB infection; (2) squares represent control foals that developed pneumonia after IB infection (n = 8); and 3) circles represent foals that were gavaged with live, virulent *R. equi* at ages 2 and 4 days (n = 5; none of these foals developed pneumonia). Fecal concentrations of virulent *R. equi* were significantly lower among foals born before April than for foals born in April or May

significantly ($P = .003$) among groups. All healthy control foals (n = 8) were born in April or May, whereas only 1 of the 8 pneumonic control foals was born in April or May; 3 of the gavaged foals were born in April or May and 2 were born in February or March. Considering birth month as a binary outcome (January, February, or March vs April or May), the fecal concentrations of virulent *R. equi* were significantly ($P = .03$) higher before challenge for foals born in April or May (median, 146 copies/100 ng DNA; range, 24 to 30 722 copies/100 ng DNA) than for foals born in January, February, or March (Figure 2; median, 30 copies; range, 0 to 6189). Excluding the 5 foals gavaged with LVRE, fecal concentrations of virulent *R. equi* were still significantly ($P = .02$) higher for foals born in April or May (median, 139 copies/100 ng DNA; range, 24 to 7841 copies/100 ng DNA) than for foals born in January, February, or March (median, 37 copies; range, 0 to 5952 copies/100 ng DNA).

4 | DISCUSSION

Virulent *R. equi* are widespread in their environment at horse-breeding farms, but only a small proportion of foals develop rhodococcal pneumonia.[1-14] Environmental exposure contributes to varying risk of disease. Airborne concentrations of virulent *R. equi* were higher at Australian Thoroughbred breeding farms that had greater cumulative incidence of *R. equi* pneumonia,[7] and airborne concentrations of virulent *R. equi* were higher in stalls of foals that subsequently developed pneumonia in Kentucky and Texas.[10,11] These findings are consistent with experimental evidence that IB/intrapulmonary infection of foals with higher numbers of CFU of virulent *R. equi* induces more frequent and more severe pneumonia.[31-33] But the relationship between natural intraintestinal exposure to virulent *R. equi* and risk of pneumonia is uncertain. Gavage of newborn foals with large numbers of virulent *R. equi* has been demonstrated to protect them against subsequent IB infection with virulent *R. equi*,[15,16] and we replicated these findings in 5 foals (for a project investigating immune responses to intragastric administration of virulent *R. equi* to newborn foals). Foals are known to be coprophagic.[34,35] Moreover, they may swallow inhaled or expectorated *R. equi*. Thus, it is plausible that young foals that ingest large numbers of virulent *R. equi* from their dam's feces or other environmental exposures are partially or fully immunized against *R. equi*. We used fecal swabs collected from a convenience sample of foals from ongoing projects to test this hypothesis and found evidence that higher fecal concentrations at age 28 days immediately before IB infection were associated with decreased likelihood of pneumonia both in gavaged foals and healthy control foals. It was not surprising that the foals gavaged with virulent *R. equi* at age 2 and 4 days were protected against pneumonia and had relatively high fecal concentrations of *vapA* at age 28 days. To our knowledge, however, these are the first data demonstrating an association between higher fecal concentrations of *R. equi* associated with lower risk of rhodococcal pneumonia in foals. Although experimental infection of foals offers advantages of controlling crucial conditions of the challenge (eg, age infected and IB infection dose), further testing of this hypothesis under natural conditions is needed. The data reported here substantiate the need for further investigation of the gut-lung axis in the context of immunity and prevention of rhodococcal pneumonia.

Two weeks after infection, fecal concentrations had increased for pneumonic foals. This is consistent with prior findings that fecal concentrations of virulent *R. equi* are significantly higher among foals with pneumonia than among foals that remain healthy, including foals with subclinical pneumonia.[21,23-27] This has been attributed at least partially to sick foals swallowing large numbers of *R. equi* in expectorated mucopurulent material. It is also possible that the intestinal microbiota of susceptible foals is more permissive to colonization or replication with virulent *R. equi*. The fecal concentration of virulent *R. equi* in the

healthy controls rose from a mean of 280 copies/100 ng DNA (95% CI, 29 to 2552 copies/100 ng DNA) before IB infection at age 28 days to a mean of 4116 copies/100 ng DNA (95% CI, 433 to 39 102) at age 42 days, but this nearly 15-fold increase was not significant ($P = .06$) and was considerably smaller (>1 order of magnitude) than that for the foals that became sick. This difference might be because the healthy control foals were either not expectorating and swallowing as many *R. equi* as pneumonic foals or had an intraintestinal environment that was less hospitable for colonization and replication of virulent *R. equi* than the pneumonic control foals.

The foals that were gavaged with *R. equi* had a marked decrease in their fecal concentrations of virulent *R. equi* after IB infection. To our knowledge, this finding has not been reported previously. Thus, it will be important to determine whether this result is reproducible. This finding suggests that intragastric administration of LVRE leads both to clearance of IB infection and to reduction in intraintestinal colonization or replication of virulent *R. equi*. It is unclear whether this decrease in fecal concentration might occur in foals gavaged with LVRE but that are not infected IB.

Birth-month, development of pneumonia, and fecal concentrations of virulent *R. equi* before challenge were coassociated. Foals that were gavaged were nearly evenly distributed between birth-months of January, February, and March (n = 2) and April or May (n = 3); however, control foals that developed pneumonia were predominately born in January, February, or March (88%; 7/8) whereas healthy control foals were born in April or May (100%; 8/8). Because of this complete separation (ie, all healthy control foals born in April or May) and our small sample size, it was impossible to separate the effects of birth-month from disease status. Fecal concentrations at age 28 days (before IB infection) were higher among foals born in April or May than earlier months, whether considering all 3 groups of foals or just the 2 groups of control foals (ie, healthy controls versus pneumonic controls). The reason for this association is unknown, but increased density of mares and foals during the later birth-months might have resulted in higher fecal concentrations of virulent *R. equi* in the environment of mares and foals.[8,18,19] Alternatively, the warmer weather in Texas in April and May might have resulted in conditions that favored growth of *R. equi* in feces and soil. Replication of these findings and further evaluation of questions arising from our results are warranted.

This study had limitations. First, the sample size was small and determined by convenience: we collected fecal samples from foals used for 2 unrelated projects. Future studies should be designed specifically to test the hypotheses examined here; our results provide data for sample size calculations for planning such studies. We used only ultrasonography to characterize pulmonary lesions. Because thoracic ultrasonography cannot penetrate a healthy lung surface, we cannot be certain that some of our healthy foals had subclinical lesions in deeper lung parenchyma which was covered by healthy lung that precluded sonographic detection of lesions. Similarly, we only monitored foals for clinical and hematological signs of disease and ultrasonographic evidence of pneumonia. It is possible that some of the healthy foals with high fecal copy numbers of *vapA* had extrapulmonary infections such as intra-abdominal lymphadenitis or enterocolitis. We think the likelihood of this is low because daily clinical examinations and biweekly hematological findings were within reference ranges for healthy control foals, and all healthy control foals were observed to have gained weight each week to a greater extent than pneumonic foals (data not provided). Nevertheless, we cannot exclude the possibility that some of the healthy foals had subclinical extrapulmonary manifestations of *R. equi* infection.[36] We did not provide evidence to substantiate that 1 copy of *vapA* represents 1 colony forming unit of *R. equi*. However, it is generally assumed because of the large size of the plasmid encoding VapA (approximately 85 kb) that each bacterium harbors only a single copy. Fecal samples were collected on a specific day for the gavaged and healthy control foals, but was variable in pneumonic foals. But all pneumonic control foals except 1 had fecal swabs collected between 12 and 16 days after IB infection, and exploratory data analysis (data not shown) did not suggest any association between age of sample collection and fecal concentration of *vapA*.

In conclusion, higher fecal concentration of virulent *R. equi* before challenge was associated with protection against IB infection with *R. equi*. These findings suggest that variability in susceptibility of foals to natural infection might in part be mediated by the numbers of intraintestinal virulent *R. equi* to which foals ingest from their environment by mechanisms such as coprophagia, pica, or swallowing inhaled or expectorated airborne organisms. After IB infection, fecal concentrations increased markedly in foals that developed pneumonia, and dramatically decreased in foals gavaged with LVRE at age 2 and 4 days. Whether gavage with LVRE results in enhanced clearance of *R. equi* from both the respiratory and intestinal tracts and the mechanism of this clearance merits further investigation.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

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 (Protocols #2020-0273 and 2020-0280).

[Correction added on 29 Apr 2022, after the first online publication: Acknowledgment section has been updated]

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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