EPIDEMIOLOGY OF AIRBORNE VIRULENT *Rhodococcus equi* AT HORSE BREEDING FARMS

A Thesis

by

KYLE RYAN KUSKIE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Noah Cohen
Committee Members,	Morgan Keith Chaffin
	Sara Lawhon
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ABSTRACT

Epidemiology of Airborne Virulent *Rhodococcus equi* at Horse Breeding Farms.

(December 2011)

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Chair of Advisory Committee: Dr. Noah Cohen

Rhodococcus equi causes severe pneumonia, resulting in disease and sometimes death of foals. Infection is thought to occur by inhalation of dust contaminated with virulent *R equi*. A recent study of 3 horse breeding farms in Ireland found airborne concentrations of virulent *R equi* to be significantly higher in stables than in paddocks. More importantly, another study from Australia established an association of airborne concentrations of virulent *R equi* with the prevalence of *R equi* pneumonia at 28 farms. The extent to which these associations extend to other farms in different parts of the world is not known.

Two farms in central Kentucky with recurrent *R equi* pneumonia in foals were studied from February through July 2008. Air samples were collected and environmental factors were measured hourly for a 24-hour period each month from stalls and paddocks used to house mares and their foals at each farm. In 2009, samples were collected from 47 foals from stalls at a single horse-breeding farm in central Kentucky on days 1-2, days 7-9, and days 14-16 of life. Concentrations of airborne virulent *R equi* were determined via a modified colony immunoblot technique.

Airborne concentrations of virulent *R equi* were significantly higher ($P = 0.016$) from 6:00 A.M. through 11:59 P.M. than for the period from midnight through 5:59 A.M. Presence of the mare and foal at the time of sampling was significantly ($P < 0.001$) associated with increased airborne concentrations of virulent *R equi* in stalls. The presence of virulent *R equi* in stalls was significantly ($P = 0.045$) more likely at 7 days of age for foals subsequently found to be affected by rhodococcal pneumonia.

These findings suggest that recovery of airborne virulent *R equi* is less likely between 12:00 A.M. and 5:59 A.M., relative to other times, that airborne concentrations of virulent *R equi* are significantly increased when horses are present at the site for collection of air samples, and that environments containing airborne virulent *R equi* during the first week of life may influence the risk of subsequent disease for a foal.

DEDICATION

I dedicate this manuscript to my parents, Kendall and Mary Jo, and also to my sister, Kristin, for all of their unconditional love and support throughout all of my endeavors past, present, and future.

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CHAPTER I

INTRODUCTION

The gram-positive, facultative intracellular bacterium *Rhodococcus equi* causes severe bronchopneumonia and suppurative lymphadenitis in foals, resulting in high morbidity and occasionally death.[1-3] Typically, *R equi* pneumonia develops when foals are 1 to 3 months of age; in most cases, infection is thought to occur earlier in life when their immune systems are relatively immature.[1] Immunity to *R equi* is complex and not fully understood, involving innate and adaptive immune responses, including both antibody- and cell-mediated processes.[4]

Rhodococcus equi pneumonia greatly burdens the equine breeding and racing industries; foals affected by *R equi* pneumonia are less likely than their birth cohort to start a race,[5] and breeding farms known to have a problem with *R equi* pneumonia often suffer a loss of clients. The pattern of occurrence at farms varies, with some farms being absent of foals affected by *R equi* pneumonia, others experiencing cases of the disease only sporadically, and some having regularly recurrences of affected foals (endemic).[2,6]

Virulence is attributed to an 85- to 90-kb plasmid with a pathogenicity island encoding at least 1 gene product, virulence-associated protein A (VapA).[7] *Rhodococcus equi* is considered a soil saprophyte and its growth requirements are easily satisfied by

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This thesis follows the style of *American Journal of Veterinary Research*.

warm climates and herbivore manure.[2,8] Epidemiological studies have demonstrated the ability to recover virulent and avirulent *R equi* from soil in paddocks and stables at horse breeding farms worldwide.[8-11] In one study,[11] soil samples from 38 farms in central Kentucky were found to contain virulent *R equi*, irrespective of the farm history of *R equi* foal pneumonia. Moreover, the investigators failed to establish an association between the concentration of virulent *R equi* in the soil and the incidence of *R equi* foal pneumonia at these 38 farms.[11] These data indicate exposure to virulent *R equi* is widespread in the environment and are consistent with a study from Australia in which soil concentration of virulent *R equi* also was not significantly correlated with the incidence of *R equi* pneumonia.[10] Other studies have shown that feces from mares and foals are an important source virulent *R equi* for the surrounding environment and possibly a direct source of exposure for foals.[7,8,12-15] One of these studies also failed to establish a relationship between the concentration of virulent *R equi* in the feces and the incidence of *R equi* foal pneumonia.[14] Molecular epidemiologic studies provide evidence of considerable genetic variability among isolates of *R equi* recovered from affected foals or soil samples from different farms and different regions.[16-18] In one study,[18] 83 *R equi* isolates obtained from tracheal secretions, nasal swabs, and soil samples at a farm were subjected to pulsed-field gel electrophoresis, dividing all the isolates into 3 major and 3 minor groups. Despite these findings, the source of infection for foals remains undetermined. It also remains unclear why some breeding farms have foals affected with *R equi* pneumonia whereas others remain unaffected, and why some foals become affected while other foals in the same environment remain unaffected.

When trying to understand the pathogenesis of an infectious disease, it is informative to examine the effects of the agent, environment, and host, and their interactions. It is thought that pulmonary infection in foals results from inhaling soil-derived virulent *R equi* that have been aerosolized.[9,19] A recent important epidemiological study[10] documented a significant association between airborne concentrations of virulent *R equi* at Thoroughbred breeding farms in Australia and the cumulative incidence of *R equi* pneumonia at those farms. The same investigative team also reported that airborne concentrations of virulent *R equi* were higher in stables/barns than in paddocks at 3 farms in Ireland.[20] These findings are important for 3 reasons: (1) it is plausible that exposure to high concentrations of airborne virulent *R equi* could contribute to increased incidence of disease at horse breeding farms; (2) foals may be at a higher risk for exposure to virulent *R equi* in stables or barns than if they were in a paddock; and, (3) monitoring airborne concentrations of virulent *R equi* could aid in the control or prevention of *R equi* pneumonia at horse breeding farms.

To the investigators' knowledge, no studies of airborne concentrations of virulent *R equi* have been reported from North America, nor has any study investigated exposure to airborne virulent *R equi* at the level of the individual foal. The purpose of this study was to determine if the associations identified by previous studies[10,20] were repeatable at horse breeding farms in North America. The objectives of this study were 4-fold: (1) to determine whether airborne concentrations of virulent *R equi* were associated with location for air sample collection on the farm (stall vs paddock), location where mares and foals were predominantly housed at the time of collection of air samples (stall vs

paddock), time of day, month, ambient temperature, relative humidity, and wind speed; (2) to determine whether a more central or a peripheral location of a stall in the barn would impact airborne concentrations of virulent *R equi* in stalls; (3) to determine whether the magnitude of exposure to virulent *R equi* in stalls and paddocks for individual foals at selected ages during early life was significantly associated with the risk of subsequently developing *R equi* pneumonia at a farm with recurrent history of this disease; and, (4) to examine the association of the airborne concentration of *R equi* with presence of the mare and foal at the sampling site during sample collection.

These objectives are important in helping to bridge gaps in the understanding of the epidemiology of *R equi* foal pneumonia. While the recently reported studies[10,20] from Australia and Ireland have shown significant associations, the extent to which these findings are repeatable in other settings is unknown. Because *R equi* foal pneumonia is observed worldwide, it is important that studies be conducted in a similar manner in other countries to help evaluate any climactic or environmental conditions that may affect airborne concentrations of virulent *R equi* and the incidence of *R equi* pneumonia. Moreover, it is important to determine the extent to which the initial studies of airborne concentrations of virulent *R equi* are consistent within and among farms. This study also examines the exposure of airborne virulent *R equi* at the individual foal level, to try and establish the risk associated with exposure early in life to airborne virulent *R equi* for subsequent development of *R equi* pneumonia later in life.

CHAPTER II

EFFECTS OF LOCATION FOR COLLECTION OF AIR SAMPLES ON A FARM AND TIME OF DAY OF SAMPLE COLLECTION ON AIRBORNE CONCENTRATIONS OF VIRULENT *Rhodococcus equi* AT TWO HORSE BREEDING FARMS*

Introduction

Rhodococcus equi, a facultative intracellular organism, is an important cause of pyogranulomatous pneumonia and lymphadenitis in foals of 1 to 6 months of age that results in high morbidity and even death.[1,13] Virulent organisms possess and 85- to 90-kilobase plasmid that encodes virulence-associated protein A, which is necessary but not sufficient to cause disease in foals.[7,21,22]

The pattern of *R equi*-induced pneumonia among foals varies among breeding farms from absent to sporadic to regularly recurrent (endemic).[2,6] Although investigators in other studies[11,14] have detected *R equi* in soil and feces of mares during the periparturient period at horse breeding farms, the principal source of exposure for foals remains undetermined. It is widely speculated that pulmonary infection results from inhalation of soil-derived virulent *R equi* found in dust from a contaminated

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*Reprinted with permission from "Effects of location for collection of air samples on a farm and time of day of sample collection on airborne virulent *Rhodococcus equi* at two horse breeding farms" by Kuskie KR, Smith JL, Wang N, Carter CN, Chaffin MK, et al., 2011. *American Journal of Veterinary Research*, 72, 73-79, Copyright [2011] by American Veterinary Medical Association.

environment.[9,10,20] Evidence from studies[20,23] conducted on other continents indicates that airborne concentrations of virulent *R equi* are positively correlated with the incidence of pneumonia attributable to infection with *R equi*. To the authors' knowledge, studies of airborne concentrations of *R equi* at horse farms in North America have not been reported.

In the study reported here, airborne concentrations of virulent *R equi* in stalls and paddocks at 2 horse breeding farms in central Kentucky were analyzed. The primary objective of the study was to determine whether airborne concentrations of virulent *R equi* were associated with any of several factors (location for air sample collection on the farm [stall vs paddock], location where mares and foals were predominantly housed at the time of collection of air samples [stall vs paddock], time of day, month, ambient temperature, relative humidity, and wind speed). As a secondary objective, we investigated whether a more central or a peripheral location of a stall in the barn would impact airborne concentrations of *R equi* in stalls.

Materials and Methods

Sample population. Two Thoroughbred breeding farms in central Kentucky with a history of recurrent *R equi*-induced pneumonia in foals (ie, *R equi*-endemic farms) were used in the study. The rationale for collection of air samples at 2 endemic farms was based on the assumption that airborne concentrations are positively associated with incidence of disease, such that use of *R equi*-endemic farms would provide relatively fewer samples that yielded negative results (ie, no growth of *R equi*).[20]

Collection of air samples. Air samples were collected monthly from February through July 2008 at each of the 2 farms from a single centrally located stall in a barn that housed mares with their newborn foals and a single outdoor paddock used to maintain mares and foals housed in that barn. Air samples were collected hourly for a 24-hour period from each location at each farm during the first week of each month. Air samples from the paddock were collected within 10 m of the gate because mares and foals tended to congregate in that area.

To test the null hypothesis that the airborne concentrations of virulent *R equi* did not differ significantly as a result of ventilation, additional samples were collected at each farm at a single time point (3:00 P.M.) each month from 2 central stalls (ie, stalls most centrally located in the barn and farthest from a main entrance or any other major opening of the barn), 2 peripheral stalls (ie, stalls located closest to a main entrance or other major opening of the barn), and 2 paddocks used for housing foals. The location for collection of air samples in these 2 paddocks was within 10 m of the gate because foals tended to congregate in that area.

A portable, commercially available air-sampling device[a] was used for collection of air samples. Culture plates (100-mm) containing modified NANAT agar medium, a selective medium commonly used for epidemiological studies of *R equi*,[12,14] were used during sample collection. The air-sampling device was placed on the ground to collect air at a height of approximately 10 cm above the floor of a stall or the ground of a paddock. For each sample collection, 500 L of air was aspirated at a rate of 100 L/min onto a culture plate containing modified NANAT agar medium. Before each sample was

collected, the sieve of the air sampling device was disinfected with an isopropanol wipe.[23] Ambient temperature, relative humidity, and wind speed were recorded by use of a handheld device[b] at the time of each sample collection. At all sample collection times, horses were predominantly maintained in stalls in the barn or in paddocks. When each sample was collected, it was recorded whether mares and foals were predominantly (ie, >80% of the mare and foal population for the barn) housed in stalls or paddocks at the time of sample collection; however, presence of a mare or foal in a particular stall at the time of sample collection was not recorded.

Modified colony immunoblot assay. Culture plates were chilled with icepacks and transported in insulated containers to the Equine Infectious Disease Laboratory at Texas A&M University, where the plates were incubated at 37°C for 48 hours. After incubation, plates were analyzed for evidence of *R equi*, which were detected on the basis of morphological characteristics. The *R equi* were then tested for virulence.

The concentration of virulent *R equi* in each air sample was determined by use of a modified immunoblotting technique for detection of virulence-associated protein A.[14,24] This method allowed for the quantification of virulent *R equi* within a background of bacterial and fungal contamination. Nitrocellulose membranes[c] were placed onto previously incubated culture plates that had at least 1 CFU of *R equi* on modified NANAT agar medium; membranes were allowed to become completely saturated. Once saturated, membranes were removed and air-dried for 30 minutes at 22°C and then heated in a hybridization oven[d] at 100°C for 1 minute. Membranes then were incubated

in a 5% nonfat dry milk[e] solution diluted in TBSS[f] at 37°C for 1 hour to block unbound sites. The nitrocellulose membranes were then washed 3 times (10 min/wash) in TBSS with 0.05% Tween 20.[g] Membranes were incubated overnight at 4°C on a rocking platform[h] with the monoclonal antibody (provided by one of the authors [ST]) diluted 1:10,000 in 5% nonfat dry milk. Membranes underwent 3 washes (10 min/wash) with fresh TBSS at 37°C. Horseradish peroxidase-conjugated goat IgG fraction against mouse IgG[i] (diluted 1:2,000 in 5% nonfat dry milk) was added to the membranes, which were then incubated for 1 hour at 37°C. Membranes underwent 3 washes (10 min/wash) with fresh TBSS at 37°C, 1 wash with citrate-EDTA buffer (10mM sodium citrate[j] and 10mM EDTA[k] [pH, 5.0]) for 5 minutes at 37°C, 1 wash with citrate-EDTA buffer and 1% dextran sulfate[l] for 10 minutes at 37°C, and 3 washes (5 min/wash) with citrate-EDTA buffer at 37°C. The substrates 3,3',5,5'-tetramethylbenzidine[m] and hydrogen peroxide were added to the membranes, which were then incubated at 37°C for 30 minutes or until there was sufficient development of the positive control membrane. The substrate was discarded, distilled water was added to stop development, and virulent *R equi* colonies were determined as those that appeared blue. Colonies of avirulent *R equi* and contaminant bacteria remained colorless.

Airborne concentrations of virulent *R equi* were calculated by use of the following equation[25]:

$$C = (T \times 1{,}000)/(t \times F)$$

where *C* is the airborne concentration of virulent *R equi*, *T* is the total number of virulent *R equi* colonies counted on the membrane, *t* is the total sample collection time, and *F* is the rate of airflow for the sampling device.

Each batch of colony immunoblots contained a positive and negative control specimen. The positive and negative control specimens consisted of pure cultures of virulent (ATCC strain 33701) and avirulent (ATCC strain 33703) *R equi*, respectively, grown on culture plates that contained modified NANAT agar medium. The pure culture strains had been grown in *R equi* minimal media,[26] and 10-fold serial dilutions were performed by use of PBS solution.[n] One hundred microliters of the dilution containing approximately 10^4 colony forming units (CFUs) of *R equi*/mL was plated for each strain. Colony immunoblot control plates were incubated under the same conditions as for the airborne sample plates.

Data analysis. The airborne concentration of virulent *R equi* represented counts and consisted of values of zero and nonzero positive integers. Because most air samples yielded no isolates of virulent *R equi*, the data set contained a large proportion of zero values. Consequently, we used a zero-inflated negative binomial (ZINB) model to analyze the airborne concentration. The ZINB model was a mixture of 2 components: an NB distribution for the unbounded counts, and a point-mass at zero to account for the inflated number of zeros. The negative binomial (NB) component was chosen because of its enhanced flexibility relative to the commonly used zero-inflated Poisson model[27] in the sense that the NB model can be viewed as a Poisson model with its rate parameter

following a gamma distribution. That is, the NB model allows more heterogeneity in the distribution of nonzero counts than does the Poisson model. We modeled the 0-versus-1 mixture by a logistic regression model in which we let the mean of the NB distribution be a linear combination of the variables under investigation. Analyses were performed by use of an expectation-maximization algorithm for mixture modeling, as described as a general method.[28] All coding was performed by use of a computing platform.[o] Values of $P < 0.05$ were considered significant.

Comparisons of the dichotomous outcome (presence or absence of virulent *R equi* in airborne samples) with location (central stall, peripheral stall, or paddock) were made by use of χ^2 or Fisher exact tests with values of $P < 0.05$ considered significant. The rationale for use of the dichotomous outcome was that most air samples yielded no isolates of virulent *R equi* (ie, data had many zeros) and that the statistical power for ZINB modeling was greatly restricted by the small sample size for these comparisons.

Results

Air samples. Of 576 possible air samples, 531 (92%) were collected to evaluate effects of location for air sample collection on the farm, housing of horses at the sample collection location, time of day, humidity, temperature, and wind speed. Forty-five (8%) samples were unavailable (10 samples were affected because of battery failure of the air-sampling device, 19 samples were damaged during shipping to the Equine Infectious Disease Laboratory, and 16 were not useable because of overgrowth of fungal contaminants). Results of descriptive statistics and ZINB analysis indicated that data

were similar and did not differ significantly between the 2 farms. Consequently, data from the 2 farms were combined for subsequent analysis.

Location effects. Effects of location for air sample collection (paddock vs stall) were significantly affected by whether horses were being housed predominantly in the barn at the time of sample collection (Table 5 – Appendix A). Concentrations typically were higher in paddocks than in stalls, although the difference was not significant (Table 6 – Appendix A). The effects of housing of horses when samples were collected significantly ($P = 0.017$) affected airborne concentrations of virulent *R equi*. When horses were predominantly housed in stalls in the barn, airborne concentrations of virulent *R equi* were higher in samples collected from stalls, and when horses were predominantly housed in paddocks, airborne concentrations of virulent *R equi* were higher in samples collected from paddocks. For example, the proportion of stalls that yielded concentrations of ≥ 4 CFUs/m^3 was 16 of 176 (9%) when horses were predominantly housed in stalls, compared with 1 of 95 (1%) when horses were not predominantly housed in stalls. In contrast, the proportion of paddocks that yielded concentrations of ≥ 4 CFUs/m^3 was 4 of 170 (2%) when horses were predominantly housed in stalls, compared with 5 of 90 (6%) when horses were not predominantly housed in stalls. There were no significant differences between farms in magnitude of the effects or the number of significant differences with regard to location for air sample collection (stall vs paddock) or location where horses were predominantly housed (stalls vs paddocks) on airborne concentrations of virulent *R equi*.

Time of day. On the basis of exploratory data analysis, time of day was categorized into 4 periods (midnight to 5:59 A.M., 6:00 A.M. to 11:59 A.M., noon to 5:59 P.M., and 6:00 P.M. to 11:59 P.M.). Compared with concentrations of virulent *R equi* for the period from midnight to 5:59 A.M., concentrations typically were higher at other time periods during the day and were significantly ($P = 0.016$) higher for the period from 6:00 P.M. through 11:59 P.M. (Table 6 – Appendix A), as determined by use of multivariate methods to adjust for the effects of location for air sample collection, the location where horses were predominantly housed, and the interaction of these 2 terms.

Month. Samples were collected monthly between February and July 2008. There was no significant effect of month on airborne concentrations, after adjusting for effects of location for air sample collection, the location where horses were predominantly housed, and the interaction of these 2 terms.

Humidity, ambient temperature, and wind speed. Humidity, ambient temperature, and wind speed were recorded at the time each air sample was collected. There were no significant effects of humidity, ambient temperature, or wind speed on the airborne concentrations after accounting for effects of location for air sample collection, the location where horses were predominantly housed, and the interaction of these 2 terms (Table 6 – Appendix A). A term for the interaction of wind speed with location for air sample collection was forced into the model a priori because of our belief that wind

speed might be more likely to influence results in paddocks than in stalls (which were more protected from effects of wind). Results were similar with respect to the magnitude of effects and the number of significant differences when the interaction term was not included and whether wind speed, humidity, and temperature were considered separately as covariates.

Central stalls, peripheral stalls, and paddocks. Additional samples were collected monthly at a single time point (3:00 P.M.) from 2 peripheral stalls, 2 central stalls, and 2 paddocks used for foals at each farm in an effort to assess the extent to which greater ventilation influenced airborne concentrations of virulent *R equi*. Of 72 possible air samples, 68 (94%) were collected and available for testing. Of these 68 samples, 11 (16%) yielded positive results for virulent *R equi*. The frequency of air samples with positive results for virulent *R equi* did not differ significantly on the basis of sample collection site (Table 7 – Appendix A). The distribution did not differ significantly between farms (number of samples with positive results for virulent *R equi* in air samples collected from central stalls, peripheral stalls, and paddocks were 2, 3, and 1, respectively, at one farm and 2, 1, and 2, respectively, at the other farm). The 75th percentile of counts of virulent *R equi* from air samples was 0 for all 3 sites at both farms. The maximal value was 10 CFUs/m^3 for samples from central stalls, 16 CFUs/m^3 for samples from peripheral stalls, and 8 CFUs/m^3 for samples from paddocks.

Discussion

Because it is generally accepted that inhalation of dust contaminated with virulent *R equi* is the primary route of infection for foals that develop pneumonia caused by this bacterium, studies conducted to evaluate airborne concentrations of virulent *R equi* can advance our understanding of the epidemiology of pneumonia attributable to *R equi* at horse breeding farms. Following up on the seminal studies[10,20] in which investigators evaluated airborne concentrations of *R equi* at horse farms in Australia and Ireland, the purpose of the study reported here was to provide data regarding the effects of time of day, location for collection of air samples on a farm, and environmental or climactic conditions on airborne concentrations of virulent *R equi* at horse breeding farms in a region of North America where horse breeding is an important industry.

Airborne concentrations of virulent *R equi* were higher in stalls than in paddocks at 3 breeding farms in Ireland.[20] In the study reported here, association of the airborne concentration of virulent *R equi* with housing location (stall vs paddock) was dependent on where horses were being predominantly housed. When horses were housed predominantly in stalls in the barn, airborne concentrations of virulent *R equi* were significantly lower in paddocks than in stalls; conversely, when horses were not being housed predominantly in the barn, airborne concentrations of virulent *R equi* were higher in the paddocks. The reason for this effect (ie, significant interaction of location for collection of air samples and where horses predominantly were housed) was not

determined, but it might be explained by the physical activity of mares and foals near the sample collection site causing more material from the environment, including virulent *R equi*, to become aerosolized or airborne. Additional studies are needed to test the hypotheses that increases in the density of horses or the amount of activity that disrupts the ground surface result in increased airborne concentrations of *R equi*. Conceivably, results of these studies may partly explain the observed positive association between cumulative incidence of *R equi*-induced pneumonia in foals and the density of horses or foals at breeding farms.[11,29] An important limitation of the study reported here was that we failed to record whether mares and foals were present in the stall at the time the air sample was collected.

We did not detect significant differences between farms for the association of concentration of airborne *R equi* with location for collection of air samples on the farm, whether horses were housed predominantly in stalls, or the interaction of those 2 terms. This was not surprising because the sample population was 2 farms that had recurrence of foals affected with pneumonia attributable to *R equi*. These farms were similar in regard to number of hectares (ie, acreage), number of horses, and management practices and were in close proximity to each other in central Kentucky. Future studies should examine a larger number of farms to determine the extent to which the findings of the study reported here are relevant to farms that differ with regard to number of horses, management practices, and geographic location.

Time of day appeared to influence the airborne concentration of virulent *R equi*. We used the period from midnight to 5:59 A.M. as the reference period; airborne

concentrations of virulent *R equi* typically increased during the day and were significantly ($P = 0.016$) higher during the period from 6:00 P.M. to 11:59 P.M. than during the reference period; there were no significant differences in airborne concentrations of virulent *R equi* among other time periods. The reason for the lower concentrations during the early morning hours is unknown, but it might be explained by increased activity of people (eg, cleaning stalls, sweeping barn aisles, and moving horses to and from paddocks) and horses in the areas of sample collection during the 3 later time periods. From the standpoint of epidemiological studies, it appears that sample collection between 6 A.M. and midnight is preferable for recovering *R equi* and that marked variation in results for samples collected during regular working hours would be unlikely. From the standpoint of disease control, it might be possible to reduce exposure to foals to virulent *R equi* by reducing activities of people (eg, sweeping aisles or cleaning stalls) in barns (or paddocks) when foals are in these areas.

Month of sample collection did not significantly affect airborne concentrations of virulent *R equi*, either when considering month as the sole variable used for a model or when adjusting for location for air sample collection, horses being housed predominantly in the barn, and time of day. In Australia, airborne concentrations of virulent *R equi* were higher during the later (and warmer) months of a 6-month study[10] during foaling season. The reasons for this discrepancy between results for that study and the study reported here are unclear, but they may be related to the relatively modest magnitude of the observed effects from Australia (estimated odds ratios of approx 2, with lower bounds of the 95% confidence intervals of those odds ratios of approx 1.2 and 1.4) and the

considerably smaller scope of the present study (which involved only 2 farms, rather than the 22 farms in Australia) that limited statistical power. Further evaluation of the effect of month on the airborne concentration of virulent *R equi* is needed.

During March 2008, one of the farms housed all its mares and foals in paddocks and completely cleaned and disinfected the barn because of diarrheal disease in foals attributed to a clostridial agent. Interestingly, only 1 of 24 (4%) air samples collected from stalls at this farm yielded virulent *R equi*, and the concentration for the sample with positive results was 2 CFUs/m^3. This distribution of samples with positive results appeared markedly lower than the cumulative distribution for stalls (Table 5 – Appendix A). Moreover, the numbers of nonrhodococcal bacteria growing on these 24 plates appeared to be fewer than those seen during any other months. This finding suggests that farms could substantially decrease airborne concentrations of bacteria, and possibly the incidence of multiple airborne disease, by routinely disinfecting barns. The use of decontamination of bedding or the barn (or stable) to reduce the incidence or severity of *R equi*-induced pneumonia has been proposed in another study.[20]

Analysis of results revealed no significant effects of humidity, ambient temperature, or wind speed on the airborne concentration of virulent *R equi*. This finding also was in contrast to that of other studies.[9,20] Authors in one of those studies[9] reported that the number of *R equi* isolated from the air increased on dry and windy days. However, the sample collecting technique used for that study differed markedly from the technique used for the present study, and this discrepancy in methods could have contributed to the conflicting results. In addition, investigators in that study[9] examined

the concentration of all environmental *R equi*, rather than examining concentrations of only virulent organisms. It is also possible that the range of values for humidity, temperature, and wind speed was more variable in other studies or that the sample size for the present study was too small to detect differences that were apparent for a larger number of farms throughout various regions of Australia.[10] Further evaluation of the effects of humidity, temperature, and wind speed at a larger number of farms in North America is warranted.

As a secondary objective, we hypothesized that because of differences in ventilation, stalls located more remotely from doorways or major openings to a barn may have higher airborne concentrations of virulent *R equi* than for stalls located adjacent to doorways or major openings. However, when samples collected at approximately the same time of day (ie, within 1 hour) were obtained from 2 central stalls, 2 peripheral stalls, and 2 paddocks, there were no significant differences in the frequency of samples with positive results for *R equi* on the basis of location. These data do not suggest that the risk of exposure to virulent *R equi* is related to the location where a foal is housed in the barn; however, further evaluation of this topic is warranted by use of a larger sample size and more accurate methods for assessing the ventilation of the area where samples are collected. An important limitation of this aspect of the present study was that we did not account for potential effect of whether mares and foals were predominantly housed in stalls or paddocks on the concentrations of stall location in the barn because we lacked adequate statistical power for such analysis. Although the presence of mares and foals was the same at the time of sample collection for the 2 barn locations (ie, peripheral vs

central stalls) and thus not a potential confounder, evaluating the difference between central and peripheral stalls would likely best be accomplished by collecting samples when mares and foals were predominantly housed in stalls because this would increase the yield of samples with positive results for *R equi*.

To our knowledge, this report is the first in which airborne concentrations of virulent *R equi* at horse farms in North America have been described. However, results reflect 2 farms in a particular region of the United States (ie, central Kentucky). Because farm management practices, soil characteristics, vegetation, and climate may vary significantly among regions within the United States, it is not possible to extrapolate findings of this study to other regions of the United States. Indeed, in the absence of data from other farms within the region, extrapolation of these findings even to other farms in the same region should be made cautiously.

On the basis of the study reported here, it appears that airborne concentrations of virulent *R equi* are associated with location on a farm (stall vs paddock) in a manner that depends on whether horses are predominantly housed in the location from which the sample is collected and that airborne concentrations are lowest during the period between midnight and 5:59 A.M. Further studies in North America are warranted to establish the relationship between airborne concentrations of virulent *R equi* and the cumulative incidence of disease and between airborne concentrations of virulent *R equi* and factors that influence those airborne concentrations, including the influence of the presence and amount of activity of horses and humans and the density of horses (and foals) per hectare. Results of the study reported here indicated that these associations

may be complex and that sample collection should be performed between 6:00 A.M. and midnight.

CHAPTER III

ASSOCIATIONS BETWEEN THE EXPOSURE TO AIRBORNE VIRULENT *Rhodococcus equi* AND THE INCIDENCE OF *R equi* PNEUMONIA AMONG INDIVIDUAL FOALS*

Introduction

The gram-positive, facultative intracellular, bacterium *Rhodococcus equi* is important for foal welfare and to the equine breeding industry because it causes severe pyogranulmatous pneumonia and lymphadenitis in foals.[1,2,5] Isolates capable of causing disease in foals possess an 85- to 90-kb plasmid with a pathogenicity island encoding at least one gene product, virulence-associated protein A, which is necessary for virulence.[7]

It is commonly accepted that *R equi* is a soil saprophyte,[8] but studies have shown that it is also possible to recover virulent and avirulent *R equi* from the feces of mares and foals, as well as from ambient air in paddocks and stalls at horse-breeding farms.[11,14,20,26] Current understanding of the pathogenesis of *R equi* pneumonia suggests that pulmonary infection results from inhaling soil-derived virulent organisms that have been aerosolized.[9,20,23] Although studies have identified multiple environments as possible sources for exposure to virulent *R equi*, it remains unclear why some foals

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become affected, whereas others in the same environment remain unaffected.[10,11,14,20] Studies monitoring airborne virulent *R equi* have furthered understanding of the epidemiology of *R equi* pneumonia at horse breeding farms: airborne concentrations of virulent *R equi* have been positively correlated with the cumulative incidence of *R equi* pneumonia at horse-breeding farms, and contaminated stables may pose a greater risk for infection.[10,20] Model-based[30] and observational epidemiological evidence[31] indicate that most foals become infected with *R equi* early in life. To the authors' knowledge, no studies have investigated the association between airborne concentrations of virulent *R equi* in stalls of individual foals early in life and the risk of developing *R equi* pneumonia. Thus, the primary purpose of this study was to determine whether the magnitude of exposure to virulent *R equi* in stalls and paddocks for individual foals at selected ages during early life was significantly associated with the risk of subsequently developing *R equi* pneumonia at a farm with recurrent history of this disease. Additionally, we examined the association of the airborne concentration of *R equi* with the following variables measured at the time of air sample collection: presence of the mare and foal in the stall, wind speed, ambient temperature, and humidity.

Materials and Methods

Farm and sampling criteria. In this study, samples were collected for 47 foals from a single horse-breeding farm in central Kentucky. This farm was selected on the basis of willingness to participate in the study, having a recurrent history of annual cumulative incidence of *R equi* pneumonia of >20% in at least 3 of the previous 5 years,

and lack of the use of screening tests, such as thoracic ultrasonography, for early detection of *R equi* pneumonia. The rationale for excluding farms that used screening tests was to exclude foals diagnosed with *R equi* pneumonia that did not develop clinical signs of disease because the probability of disease recorded by positive screening tests is unknown.[32]

Air sampling criteria and procedures. Air samples were collected from the stalls used to house each mare and foal at three different time points during the neonatal period: birth (ie, day 1 or 2 of the foal's life), 1 week of age (ie, 7 to 9 days of life), and 2 weeks of age (ie, 14 to 16 days of life). Samples were also collected from the paddock in which each mare and foal was maintained at 7 to 9 days and later at 14 to 16 days of life. A paddock sample was not obtained for days 1 to 2 of life because mares and foals remained in stalls during that period and because it was often uncertain at that time which paddock would ultimately be used for a given mare-foal pair. Before the foaling season, all stalls used for foaling or housing mares and foals were disinfected. Stall walls were wooden and solid; all stalls were open at the top, permitting air to pass between stalls. All samples were collected between 9 A.M. and 6 P.M. because no significant differences were observed between these periods in a previous study conducted by our laboratories.[33]

Air samples were collected using a portable, commercially available air sampling device,[a] Petri dishes measuring 100 mm and containing a modified base medium supplemented by nalidixic acid,[p] novobiocin,[q] cycloheximide,[r] and potassium tellurite[s]

(NANAT) were used for sampling. The NANAT medium is a selective growth media commonly used for epidemiological studies of *R equi*.[12,14] Samples were collected by placing the air sampler on the ground to collect air approximately 10 cm above the stall floor or paddock ground. In all, 500 L of air were aspirated onto a culture plate, at a rate of 100 L/min. Before each collection, the sieve of the air sampler was disinfected by swabbing with an isopropanol wipe.[23] Ambient temperature, relative humidity, and wind speed were also recorded at the time of collection of each air sample using another handheld device.[b] Whether mares and/or foals were present at the sampling location when a given air sample was collected was also recorded.

Modified colony immunoblot assay. Culture plates were shipped to the Equine Infectious Disease laboratory at Texas A&M University in insulated containers chilled with icepacks. After being received at the laboratory, plates were incubated at 37ºC for 48 hours. After incubation, colonies of *R equi* were identified according to morphological characteristics. Plates positive for *R equi* growth were then analyzed for virulent *R equi* by using a modified colony immunoblotting assay for detection of virulence-associated protein A.[11,14,24,33] This method allows for the quantification of virulent *R equi* within a background of bacterial and fungal contamination. Airborne concentrations of virulent *R equi* were expressed as CFU per cubic meter of air (CFU/m^3), calculated by the following equation:

$$C\ (CFU/m^3) = (T \times 1{,}000)/(t \times F)$$

where *C* is the airborne concentration of virulent *R equi*, *T* is the total number of virulent *R equi* colonies counted on the membrane, *t* is the total sample collection time, and *F* is the rate of airflow for the sampling device.[25]

Each batch of colony immunoblots contained a positive and negative control specimen. The positive and negative controls consisted of pure cultures of virulent (ATCC strain 33701) and avirulent (ATCC strain 33703) *R equi* grown on modified NANAT culture plates. The pure culture strains had been previously grown in *R equi* minimal media.[26] Colony immunoblot control plates were incubated under the same conditions and at the same time as the airborne sample plates.

Disease status data collection. In September 2009, the farm veterinarian was provided with a follow-up questionnaire for each foal, which included information about the following variables: whether the foal developed clinical signs of *R equi* pneumonia, age at and date of diagnosis of *R equi* pneumonia, outcome of disease (lived or died), methods used for determining the diagnosis of *R equi* pneumonia in the foal, and whether the foal had other forms of *R equi* infection (abdominal lymphadenitis, osteomyelitis, enterocolitis). Four of the foals enrolled in the study were omitted from the final data analysis because one foal was euthanized (because of a scrotal hernia) and 3 were transferred off the farm before all airborne samples were obtained. For the purposes of this study, at least 1 foal at the farm had to demonstrate clinical signs of pneumonia and have microbiologic culture of *R equi* from a tracheobronchial aspirate (TBA) along with cytologic evidence indicating septic pneumonia, to substantiate that

pneumonia caused by *R equi* occurred at the farm. Foals were defined as having *R equi* pneumonia if they had clinical signs of pneumonia and any one of the following diagnostic findings: (1) multifocal pulmonary opacities on thoracic radiographs, (2) ultrasonographically visible pulmonary consolidation or abscessation, (3) positive results of microbiologic culture of *R equi* from TBA fluid, and (4) cytologically visible gram-positive intracellular coccobacilli in the TBA fluid. These diagnostic criteria were determined a priori and have been used previously by the investigators.[11,14,15,33]

Data analysis. Because most air samples yielded no isolates of virulent *R equi*, the data set contained a large proportion of zeros. Consequently, a zero-inflated Poisson (ZIP) model was used to analyze the data.[27] The ZIP model is a mixture of 2 components: a Poisson distribution for the unbounded counts, and a point-mass at zero to account for the inflated number of zeros. The 0-versus-1 data were modeled by a logistic regression model, whereas the count data were modeled such that the mean of the Poisson distribution was a linear combination of the variables under investigation. The analyses were performed using the expectation-maximization (EM) algorithm for mixture modeling, as described as a general methodology in McLachlan and Peel.[28] Nested ZIP models were compared using a likelihood ratio test.[34] Non-nested models (eg, a Poisson model vs. a ZIP model) were compared using a Vuong test.[35] All coding was performed by use of a computing platform.[o] A value of $P < 0.05$ was used for significance. Proportions of air samples positive for virulent *R equi* between stalls and paddocks were compared using χ^2 analysis, and the proportion of samples collected with

mares and foals present at the time of sampling were compared between foals that went on to develop *R equi* pneumonia and foals that remained free of *R equi* pneumonia using Fisher's exact test.

Results

Location. Using ZIP modeling, the concentrations of virulent *R equi* were significantly greater in samples collected in stalls than those collected in paddocks ($P < 0.001$), even after adjusting for effects of age of the foal, disease status of the foal, and presence of the mare and foal at the time of sampling. Comparison of these proportions using χ^2 analysis also identified a significant ($P < 0.001$) difference between these 2 locations. Of the 81 samples collected in paddocks, only 6 (7.4%) yielded positive results for virulent *R equi*, whereas positive results were obtained in 40 (31.5%) of 127 samples from stalls. The median concentration of virulent *R equi* was 0 CFU/m^3 from both paddocks and stalls (range: 0 to 2 CFU/m^3 and 0 to 32 CFU/m^3, respectively). Among air samples yielding positive results for virulent *R equi*, the concentration of virulent *R equi* in each of the 6 air samples collected from paddocks was 2 CFU/m^3, whereas the median for positive samples from stalls was 4 CFU/m^3 (range: 2 to 32 CFU/m^3).

Association of age and disease status with the concentration of virulent R equi in air samples collected in stalls. Seven of the 47 foals enrolled in the study were subsequently diagnosed with pneumonia attributed to *R equi*. The median age at the time

of diagnosis was 3 months, and all of the foals had clinical signs of pneumonia and sonographic evidence of pneumonia; one foal had *R equi* recovered by microbiologic culture from joint fluid.

Because the number of paddock air samples that yielded positive results was small, and because paddock air samples were not collected at birth, only data collected from stalls were evaluated to determine the association of airborne concentration of virulent *R equi* with subsequent development of *R equi* pneumonia and age at the time of sampling. ZIP regression analysis revealed that the zero-inflation parameter (the parameter which captures extra zeros in the airborne concentration of virulent CFU) was significantly affected by age. Also, the interaction between the presence of *R equi* pneumonia and age had a statistically significant ($P = 0.045$) effect on the zero-inflation parameter (Table 8 – Appendix A). In the count data model, airborne concentrations of virulent *R equi* were significantly greater at 1 and 2 weeks of age than at birth. Neither effects of subsequent *R equi* pneumonia nor the interaction term for subsequent pneumonia and age were significantly associated with the mean airborne concentration of virulent *R equi* in the count model (Table 8, count data model – Appendix A). The crude odds of subsequent development of *R equi* pneumonia were approximately 2.5-fold greater among foals that had positive values of airborne concentrations of virulent *R equi* (ie, *R equi* detected) at 7 days of age than for foals that had zero values in stalls at the same age (Tables 8 and 9 – Appendix A). Including a term for *R equi* pneumonia in the count model of the ZIP regression did not alter the magnitude or significance of findings (Table 10 – Appendix A). A likelihood ratio test indicated that the model

including the term for *R equi* pneumonia in the count data model (Table 10 Appendix A) was not significantly ($P = 0.371$) better than the model without this term (Table 8 – Appendix A). However, the ZIP model was significantly ($P < 0.001$) better than a standard Poisson regression model (data not shown) representing the same terms.

Association of other variables with airborne concentrations of virulent R equi. A secondary aim of the study was to determine whether airborne concentration of virulent *R equi* was associated with presence of mares and foals in the stall, humidity, temperature, and wind speed (all determined at the time of sampling). ZIP modeling indicated that presence of the mare and foal was significantly associated with the concentration of airborne virulent *R equi* at the time of sampling (Table 11 – Appendix A), both for the count data model and the zero values data model (model for the zero-inflation parameter). In this modeling, age was significantly associated with the airborne concentration of virulent *R equi*, similar to what was observed in modeling to examine effects of disease (Table 8 – Appendix A). A ZIP model identical to that seen in Table 11, but which also included age in the model for the zero-inflation parameter, did not alter significance or magnitude of associations and did not significantly improve fit of the data ($P = 0.655$). Wind speed, humidity, and ambient temperature were not significantly associated with airborne concentration of virulent *R equi*, either individually or after adjusting for effects of age and presence of mares and foals at the time of sample collection.

As a result of the small number of foals that developed pneumonia attributed to

R equi, it was not possible to include a term for presence of mares and foals at the time of sampling in models examining the association of airborne concentrations of virulent *R equi* with the development of pneumonia because of the problem of complete separation (ie, some cells of stratified tabulations having no observations). Exploratory data, however, indicated that the effect of presence of mares and foals at the time of sampling would not have confounded the association between airborne concentration of virulent *R equi* and disease. For example, there were 35 foals for whom data regarding presence or absence of mares and foals at the time of sample collection was recorded at 7 days of age (30 foals that did not go on to develop *R equi* pneumonia and 5 that did). At 7 days of age, the proportion of samples collected with the mare and/or foal present at the time of sampling did not differ significantly between the 5 foals that ultimately developed *R equi* pneumonia (40%, 2/5) and among the 30 (60%, 18/30) that did not go on to develop *R equi* pneumonia ($P = 0.6313$, Fisher's exact test; Table 12 – Appendix A).

Discussion

In our study, there was a significant association between airborne concentration of virulent *R equi* and the risk of subsequent development of *R equi* pneumonia that depended on age. The observed effects of age were difficult to interpret. At 7 days of age, airborne concentrations of virulent *R equi* in stalls were significantly ($P = 0.045$) greater in the zero values data component of the ZIP mixture model for the 7 foals that later developed *R equi* pneumonia (median: 4 CFU/m^3, range: 0 to 26 CFU/m^3) than

those that did not later develop *R equi* pneumonia (median: 0 CFU/m^3, range: 0 to 32 CFU/m^3); however, this difference was not significant for the count data component of the ZIP mixture model. There was no significant association between the presence of airborne *R equi* and the subsequent development of rhodococcal pneumonia at 2 weeks of age. The importance and interpretation of these findings remain unclear. It is possible that the observed difference in the presence of airborne *R equi* at approximately 1 week of age in the stalls of foals that subsequently developed pneumonia attributed to this bacterium may have occurred simply by chance alone. Alternatively, it is possible that exposure to airborne virulent *R equi* in stalls during early life contributes to increased risk of *R equi* pneumonia. Inhalation of virulent *R equi* is considered to be the route of infection, and one study found that concentrations of virulent *R equi* in the air are positively associated with the risk of disease.[20] Clinical signs of *R equi* pneumonia are generally observed when foals are between 30 and 90 days of life,[1] but the age of infection and the incubation period remain unknown. Recent evidence suggests the hypothesis that foals become infected early in life,[30,31] rather than during the time when maternal antibodies are decreasing to their nadir.[36,37] If the observed association between airborne virulent *R equi* at approximately 1 week of age and the subsequent development of *R equi* pneumonia reported in this article is true, it is further evidence that infection may occur early in life. Although our results do not indicate any association between the magnitude of airborne concentration of virulent *R equi* (ie, no significant association in the count model) and the risk of disease, exposure to virulent *R equi* was significantly associated with the risk of disease. Exploratory data analysis (Fig. 1 – Appendix B)

suggests that the distribution of absolute concentrations of airborne virulent *R equi* on day 7 (as well as the presence or absence of the bacterium) differed between affected and unaffected foals, but the small number of affected foals may have limited statistical power to detect a true difference. A larger study is warranted to confirm or refute these results.

The concentrations of virulent *R equi* isolated from stalls were significantly ($P < 0.001$) greater in stalls than paddocks, and results indicated that the proportion of air samples yielding positive results for virulent *R equi* in stalls (31.5%) was significantly ($P < 0.001$) greater than in paddocks (7.4%). This finding is consistent with results from a previously reported study involving three horse-breeding farms in Ireland,[20] but is in contrast to the results of a previous study conducted in Kentucky by our laboratories.[33] The reason for the differences of results among studies is unclear, but could be attributable to variations among farms in factors that influence airborne concentrations in barns (eg, barn ventilation), or variation among studies with regard to the cumulative incidence of disease and criteria for defining *R equi* pneumonia on affected and unaffected farms.[11,14,23]

In our previous study,[33] data analysis revealed that horses being present at the *general* location of sampling (ie, whether horses were predominately in the barn or in paddocks at the time of sampling) significantly increased the chances of recovering virulent *R equi* from the ambient air. This finding was a serendipitous and unexpected finding because that study had not been designed a priori to determine whether presence of mares and foals influenced the results of airborne concentrations, in fact, one of the

authors (JLS) astutely recorded whether horses were predominantly in the barns or in paddocks at the time of sampling. A major limitation of that observation was that it was not known whether mares and foals were present *specifically* at the site of sampling (ie, we did not ascertain whether specific stalls were occupied by a mare or foal at the time of sampling in that study). In this study, however, we attempted to determine whether a given mare-foal pair was present in the stall at the time of sampling to better assess the association between presence of mares and foals at the time of sampling. The presence of mares and foals in the stalls was positively and significantly associated both with the absolute concentrations of virulent *R equi* in airborne samples and whether virulent *R equi* was isolated from the air ($P < 0.001$ and $= 0.046$ for the counts and zero values components of the ZIP model, respectively [Table 11 – Appendix A]). This finding is important for at least 2 reasons. First, it indicates that studies of airborne concentrations of *R equi* at horse farms must account for whether horses are present at the sampling site. Second, it may have implications of control and prevention of *R equi* pneumonia. Epidemiological studies have determined that soil and feces from the mare are important sources of exposure of foals to *R equi*, but were unable to show an association between the concentration of *R equi* in these environmental sources and the cumulative incidence of *R equi* pneumonia[11,14,15] It has been suggested previously that the stabling of mares and foals for extensive periods could increase the prevalence of disease at a farm because of a more concentrated and constant exposure to dust contaminated with virulent *R equi* from the feces of mares and foals.[9,20] Our results regarding presence of mares and foals are consistent with this suggestion, and indicate the need to evaluate the

effect on the incidence of foal pneumonia of methods to reduce either airborne *R equi* in stalls where mares and foals are housed or extent to which foals are housed in stalls where higher airborne concentrations of *R equi* may occur. Similarly, studies on the effect of increased density of horses at a farm and how the activities of horses and farm personnel contribute to increased airborne concentrations of *R equi* are also warranted.

This study is not without important limitations. First, results may only reflect the circumstances of the single farm that was studied. As previously mentioned, there seems to be variability in the results of epidemiological and ecological studies of airborne concentrations of *R equi*. Second, samples were only collected at 3 points in time during early life, limiting the extent to which it can be concluded that infection was occurring during this early neonatal period: we have no data regarding exposures at older ages. Moreover, extrapolating results from one farm to any other farm must be made with caution. Our rationale for focusing on early life is that causes must precede effects; therefore, it would be difficult to assert a causal relationship between air samples collected at later ages when foals might already have progression of clinical signs and pathological lesions, and we would be more likely to suspect that relatively higher airborne concentrations are a cause of rather than an effect of disease if the elevated concentrations precede clinical or pathological abnormalities.

Third, the moderate sample size of only 47 total foals, including only 7 that developed *R equi* pneumonia, limited our statistical power. This precluded us from being able to simultaneously examine the association of airborne concentrations of virulent

R equi with age, subsequent development of *R equi* pneumonia, and presence of mares or foals when samples were collected because of the problem of complete or near complete separation (ie, zero values in some strata of multivariate analysis). Although subsequent studies using a larger sample size will help address this limitation, we found no significant association between disease and whether mares and foals were present at the time of sampling, indicating that confounding was improbable (Table 12 – Appendix A). Another limitation of this study is that not all samples were collected in a uniform manner with respect to presence of mares or foals at the time of sample collection. Ideally, we would have collected all samples with mares or foals present (or absent) to avoid potential confounding by this factor. However, at the time the study was designed and implemented, the association between the presence of mares or foals and airborne concentrations of virulent *R equi* was not known to us and was not specified in previous studies. As a consequence, we only recorded whether mares and foals were present at the time of sampling. Moreover, we only recorded these data for 35 of 47 foals. Still, an important result of this study is that it is first to document that presence of mares or foals at the time of sample collection significantly increases airborne concentrations of virulent *R equi* in stalls. As with any farm-based study of *R equi*, the potential for misclassification of disease status exists. In this study, all foals diagnosed with *R equi* pneumonia had clinical signs of pneumonia (fever and either cough, nasal discharge, or elevated respiratory rate) and ultrasonographic evidence of pulmonary consolidation or abscessation concurrent with clinical signs; however, isolation of *R equi* by microbiologic culture of fluid recovered by tracheobronchial aspiration was only

confirmed in 1 foal. Three of the 7 foals diagnosed with *R equi* pneumonia that had clinical signs of pneumonia and ultrasonographic evidence also had elevated white blood cell concentrations, and another foal also had radiographic evidence consistent with *R equi* pneumonia. One foal was diagnosed with pneumonia other than *R equi* on the basis of findings of clinical examination, thoracic ultrasonography, microbiologic culture, and response to treatment; virulent *R equi* were not identified in air samples from the stall of this foal at any age. However, the effect of any disease misclassification cannot be predicted, but the expected effect would be to bias the association towards the null (ie, toward observing no association of disease with exposure) when the misclassification is non-differential (ie, when the proportion of foals misclassified as diseased does not depend on exposure status).[38] Selection of an appropriate model for the data was another difficult task. A Poisson model, which excluded a zero-inflation model, yielded similar results to the count model used in this study. In the Poisson model, the effects at birth and subsequent development of *R equi* pneumonia was less and not significant ($P = 0.412$, data not shown). Comparison of the models with a Vuong test indicated that a ZIP model was significantly ($P < 0.001$) better than the Poisson model. Using the zero-inflated term including both age and *R equi* pneumonia provided the best fit.

In summary, airborne concentrations of virulent *R equi* were significantly higher in stalls than in paddocks at this farm, and virulent *R equi* were more frequently isolated from air samples obtained from stalls than from paddocks. Additionally, the concentration of virulent airborne *R equi* was significantly higher in stalls when mares

and foals were present at the time of sample collection. Among the foals that later developed *R equi* pneumonia, it was more likely that virulent airborne *R equi* was present in the stall at approximately 1 week of age than for foals that remained free of this disease. Further evaluation of the potential causal relationship between airborne concentrations of virulent *R equi* in stalls of foals and subsequent risk of *R equi* pneumonia are needed to substantiate the results of this study.

CHAPTER IV

ADDITIONAL EXPERIMENTS AND CONCLUSIONS

In addition to the work described in Chapter II and Chapter III, several other experiments were conducted in relation to this project. The objective of this chapter is to summarize that work.

Media Experimentation

Beef broth supplementation. The modified NANAT agar medium was chosen as the selective media for *Rhodococcus equi* isolation in this study on the basis of its successful use in other epidemiological studies in which *R equi* was isolated from the feces of mares and foals and from soil at horse breeding farms.[6,11,12,14,15] The original formulation of this medium[12] had been previously modified[14] by adding beef broth to improve growth of *R equi* without diminishing selectivity for growth of *R equi*. However, preparation of the boiled beef broth was very time-consuming. Because of the large number of NANAT agar media plates needed for our projects, an experiment was conducted to identify an alternative to preparing boiled beef broth in our laboratory for the modified NANAT media.

For this experiment, growth of a virulent strain of *R equi* (ATCC 33701+) and an isogenic, plasmid-cured, avirulent strain of *R equi* (ATCC 33701-) were compared on modified NANAT agar media using 1 of the following modifications to the beef broth formulation: (1) 300 ml of boiled beef broth; (2) 300 ml of beef broth;[t] (3) 100 ml of low

sodium beef broth;[u] and (4) 25 g of beef extract[v] in 300 ml of ddH_2O. Pure cultures of ATCC 33701+ and ATCC 33701- had been previously grown in *R equi* minimal media[26] to a concentration of approximately 10^8 CFU/ml. Ten-fold serial dilutions (1:10) were prepared using PBS[n] and 100 µl of the dilution containing approximately 10^2 CFU/ml was plated onto each of the previously mentioned modified NANAT agar media plates, in duplicate. The plates were allowed to incubate at 37ºC for 48 hours. After incubation, the plates were removed, counted, and averages prepared; results from this experiment are summarized in Table 1.

Table 1. Comparison of growth of virulent (ATCC 33701+) and avirulent (ATCC 33701-) *Rhodococcus equi* using different beef broth formulations for NANAT agar media.

Broth Formulation	ATCC 33701+ (CFU/mL)	ATCC 33701- (CFU/ml)
Boiled Beef Broth	1.75×10^8	3.25×10^8
Beef Broth	8.85×10^7	3.05×10^8
Low Sodium Beef Broth	1.12×10^8	4.15×10^8
Beef Extract	1.05×10^8	2.60×10^8

It was determined that using the low sodium beef broth would be a suitable replacement for the boiled beef broth because it had minimal inhibitory effects on the growth of *R equi* from minimal media and minimized the amount of extra sodium included in the modified NANAT agar media formulation.

Before proceeding with the modified NANAT agar medium using a low sodium beef broth, we determined whether there were any inhibitory effects on the growth of *R equi* from environmental samples. Because an air sampling device was not yet available for testing the different modified NANAT agar media, 3 soil samples and 3 fecal samples known to contain *R equi*, from previously conducted studies,[11,14] were cultured on modified NANAT agar media with boiled beef broth and modified NANAT agar media with low sodium beef broth. One gram of soil or feces was suspended in 5 ml of PBS. Ten-fold serial dilutions (1:10) were performed using PBS and 100 µl of the 10^{-2} dilution was plated onto each type modified NANAT agar media, in duplicate. Plates were allowed to incubate at 37º for 48 hours. Following incubation, the plates were removed and colonies of *R equi* were counted on the basis of morphological characteristics; the concentration (CFU/g) was determined for each sample, and results were tabulated (Table 2).

Table 2. Growth of *R equi* in soil and feces on modified NANAT agar media using boiled beef broth or low sodium beef broth.

Sample	Boiled Beef Broth (CFU/ml)	Low Sodium Beef Broth (CFU/ml)
Soil #1	2.63×10^4	2.13×10^4
Soil #2	1.28×10^4	1.43×10^4
Soil #3	7.83×10^4	7.67×10^4
Feces #1	2.16×10^3	1.78×10^3
Feces #2	2.23×10^3	1.87×10^3
Feces #3	1.13×10^3	2.00×10^3

Results of this experiment indicate that supplementing boiled beef broth with low sodium beef broth had minimal, if any, inhibitory effect on the growth of *R equi* from environmental samples. Thus, the modified NANAT agar media prepared with low sodium beef broth instead of the boiled beef broth was used for airborne studies.

Amphotericin B. During the collection of air samples during the study conducted during 2008, it became apparent that another change to the modified NANAT agar media was necessary. This was because it appeared that the number of fungal contaminants observed on the incubated plates rose with increasing average ambient temperature in Kentucky, despite the presence of the antifungal agent cycloheximide^{r} (40 μg/ml) in the media. Mold colonies generally were white, green, or yellow, and large circles of spores would sometimes engulf the entire agar surface, making it impossible to identify bacterial colonies or transfer proteins onto the nitrocellulose membranes. Sixteen of the samples collected during this study were unusable because of this type of fungal overgrowth; the number of *R equi* colonies that were not counted, or not identified as virulent by the modified colony immunoblot assay from samples contaminated with mold is unknown. Three of the more commonly observed molds with different phenotypes were sub-cultured and delivered to the Veterinary Clinical Microbiology Laboratory at Texas A&M University for identification. Laboratory testing confirmed that all 3 molds submitted were *Aspergillus spp*. It was suggested that amphotericin B^{w} be included in the modified NANAT agar media because it has a similar antifungal spectrum but different mode of action than cycloheximide;

cycloheximide prevents protein synthesis by blocking the translation of mRNA by ribosomes whereas amphotericin B impairs the barrier function of the fungal membranes.[39]

The purpose of the first experiment conducted relevant to antifungal activity was to determine whether modified NANAT agar media containing amphotericin B could effectively inhibit the growth of the 3 *Aspergillus spp*. that were isolated from air samples taken in Kentucky. On the basis of results of a previous study[39] amphotericin B was added to the modified NANAT agar medium at a concentration of 10 μg/ml. Agar plates were prepared containing the modified NANAT agar, modified NANAT agar with amphotericin B, and modified NANAT agar with amphotericin B but without cycloheximide. Brain heart infusion[x] (BHI) agar also was prepared for use as a positive control. Colonies of each of the 3 *Aspergillus spp*. were streaked onto each of the 4 different types of agar media in duplicate; results of this experiment are summarized in Table 3.

From this experiment we determined that cycloheximide (40 mg/L) alone was not sufficient to completely inhibit the growth of the 3 *Aspergillus spp.* tested. The inclusion of amphotericin B (10 μg/ml), either as a substitute for cycloheximide or in addition to cycloheximide, in the modified NANAT agar media was sufficient to completely inhibit the growth of 2 of the *Aspergillus spp.* tested and moderately inhibit the growth of the third, when compared to growth on the BHI agar.

The purpose of the second antifungal experiment conducted was to determine whether amphotericin B inhibited the growth of *R equi* when cultured on modified NANAT agar media containing amphotericin B (10 mg/L). Eighteen plates each of

Table 3. Comparison of the growth of *Aspergillus spp.* on different agar media.

	***Aspergillus spp.* #1**	***Aspergillus spp.* #2**	***Aspergillus spp.* #3**
BHI	**+++**	**+++**	**+++**
NANAT	**++**	**++**	**++**
NANAT (+) Amphotericin B (-) Cycloheximide	**-**	**+**	**-**
NANAT (+) Amphotericin B (+) Cycloheximide	**-**	**+**	**-**

+++ Uninhibited growth
++ Slightly inhibited growth
+ Moderately inhibited growth
- Completely inhibited growth

modified NANAT agar, modified NANAT agar with amphotericin B, and modified NANAT agar media with amphotericin B but absent of cycloheximide were prepared. A virulent strain of *R equi* (ATCC 33701) was grown in minimal media[26] to a concentration of approximately 10^8 CFU/mL. Ten-fold serial dilutions (1:10) were performed using PBS and 100 µL of the dilution containing approximately 10^2 CFU/mL was spread onto each of the agar plates. The plates were allowed to incubate at 37ºC for 48 hours, colonies of *R equi* were counted, and CFU/mL was determined for each plate. Exploratory data analysis (Fig. 2 – Appendix B) suggested that the distribution of

CFU/mL of *R equi* did not differ significantly between any of the 3 different types of agar media tested. A 1-way analysis of variance (ANOVA) test was performed using a statistical software package[y] to test for any significant differences between the different types of modified NANAT agar media and results are presented in Table 4. The *P*-value of the ANOVA test was greater than 0.05 ($P = 0.967$), therefore we failed to reject the null hypothesis that there were no difference among the 3 modified NANAT media tested to support the growth of virulent *R equi* (ATCC 33701).

Table 4. One-way analysis of variance (ANOVA) test comparing the growth of virulent *R equi* (ATCC 33701) on 3 different types of modified NANAT agar media.

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	7.259 E12	2	3.630 E12	0.033	0.967
Within Groups	5.585 E15	51	1.095 E14		
Total	5.593 E15	53			

It was unknown, and nearly impossible to test, the effects of removing cycloheximide from the media would have in regards to other fungal growth. Therefore, considering the results of the first experiment (viz., that the inclusion of amphotericin B in the modified NANAT agar media would inhibit the growth of *Aspergillus spp.* of fungi) and the second experiment (viz., that the inclusion of amphotericin B in the modified NANAT agar medium did not inhibit the growth of virulent *R equi* [ATCC 33701]) the decision was made to add amphotericin B (10 µg/ml), in addition to cycloheximide (40 µg/ml), to the modified NANAT agar media used for sampling air

from stalls and paddocks at horse farms in Kentucky. As a result, none of the air samples received during the 2009 study were unusable because of fungal overgrowth.

The final formulation for the modified NANAT agar medium used for the studies of airborne virulent *R equi* consisted of the following: 100 mL low sodium beef broth; 800 ml distilled water; 20 g peptone;[z] 5 g sodium chloride;[aa] 5 g yeast extract;[bb] 5 g glucose;[cc] 0.2 g sodium dithionite;[dd] 1.2 g sodium thiosulfate;[ee] 2 g potassium phosphate;[ff] 2 g sodium bicarbonate;[gg] and 12 g agar-agar.[hh] The pH was adjusted to 7.2, final volume adjusted to 1 L, and autoclaved for 30 min at 121ºC. When the medium had cooled to 55ºC the following antibiotics were added: 20 µg/ml nalidixic acid;[p] 25 µg/ml novobiocin;[q] 40 µg/ml cycloheximide;[r] 3.5% potassium tellurite;[s] and 10 µg/ml amphotericin B.

Shipping Samples

Temperatures. In April and May of 2008, some problems arose regarding the manner in which the modified NANAT agar media plates were being shipped between Texas and Kentucky. Originally, plates were inverted and packaged into a Styrofoam ice chest that contained several ice packs to keep the plates cool. Plates were then shipped via overnight courier. However, as average temperatures began to rise through the spring months, air sample plates received by the EIDL had accumulated a great deal of condensation which would sometimes accumulate on the inverted agar. Following incubation, colony morphology was nearly impossible to determine because these plates grew a lawn of bacteria rather than individual colonies. Additionally, high temperatures

caused shrinking of the agar, resulting in the agar falling onto the lid of plates either during shipping or incubation. In addition, the higher temperatures resulted in premature growth of some bacterial colonies. Nineteen samples were lost during the 2008 airborne study as a result of these complications of shipping.

Due to the amount of time required to analyze plates for the presence of *R equi*, the amount of time required to immunoblot those plates positive for *R equi*, and the minimal number of personnel in Kentucky collaborating on this study, processing air samples at a laboratory in Kentucky was not feasible. Thus, a method for shipping plates that helped control the temperature of the plates inside the shipping containers was pursued.

After consulting several microbiologists, the following protocol was devised and implemented for the remainder of the 2008 airborne study, as well as the 2009 airborne study. Plates containing modified NANAT agar media were placed inside the plastic sleeves in which the unused plates were shipped from the manufacturer, and each sleeve was sealed shut using a metal twist tie (commonly packaged with garbage bags). Sleeves of plates were then placed inside of a soft insulated beverage cooler[ii] that was zipped shut. The insulated cooler was then placed into a large Styrofoam cooler which contained several ice packs. The Styrofoam container was placed into a cardboard box and shipped via overnight courier. This method for packaging was used both for shipping modified NANAT agar media plates to Kentucky for sample collection and shipping plates that had been used to collect samples in Kentucky to Texas. This shipping protocol remedied the problems attributed to the increased ambient temperature

and no further samples received during the 2008 or 2009 study were unusable because of excess condensation or fallen agar.

Storage of air samples. In the 2008 airborne study, samples were collected once monthly for a 24-hour period and immediately shipped via overnight courier to the EIDL. In the 2009 airborne study however, samples were collected throughout the foaling season at 1 farm on days 1-2, days 7-9, and days 14-16 of life for each foal born at the farm. We anticipated only collecting a few air samples during the early and late months of the foaling season, similar to a Poisson distribution, with the majority of samples coming in the middle of the foaling season. Because of the expense of courier shipping, it was not economical to make an overnight shipment for each day that air samples were collected, especially for days when only a few plates were collected. We thus proposed a weekly shipment of air samples from Kentucky to minimize the shipping costs for this project.

Before the start of the 2009 airborne study, a small experiment was conducted to determine the maximal duration that an air sample could be stored in a refrigerator before bacterial growth was observed. Eight air samples (500 L each) were collected in duplicate from different stalls used for housing horses at the Texas A&M University Horse Center. A plate from each location was packaged into a plastic sleeve, inverted, and stored in a refrigerator at 4ºC. The duplicate plates were immediately placed in an incubator at 37ºC and allowed to incubate for 48 hours. The refrigerated plates were examined daily for 10 days to monitor the growth of any bacteria; after 10 days they

were placed in an incubator at 37ºC and allowed to incubate for 48 hours. The growth of bacteria was then compared between the refrigerated plates and those that were incubated immediately after sample collection.

After 8 days of storage in a refrigerator, 1 plate had 3 detectable colonies and the remaining 7 plates had no visual evidence of bacterial growth. After 10 days of refrigeration, 1 additional plate had 1 colony of bacteria that was detectable; the other 6 plates remained negative for bacterial growth and the plate positive for growth on day 7 still had only 3 colonies of bacteria; however, these colonies had slightly increased in diameter. Visual comparison of the refrigerated plates after incubation against the corresponding plates that were immediately incubated showed no evidence of bacterial overgrowth or any inhibitory effects related to the delayed incubation. As a result, it was determined that storing plates in a refrigerator for 6 days before shipping would have no substantive effects on the results of the air sampling. Thus, weekly shipments of plates were scheduled so that the maximum duration before a plate was placed in the incubator was 7 days.

Conclusions

Rhodococcus equi remains a severe cause of pneumonia in foals. Epidemiological studies that help identify environmental risk factors, as well as strategies for infection control and prevention are especially important because of the lack of an effective vaccine. Recent studies from Australia have described an association

between airborne concentrations of virulent *R equi* and the prevalence of *R equi* pneumonia at horse breeding farms; additionally, airborne concentrations of virulent *R equi* in Ireland were higher in stables than in paddocks. The extent to which these important associations can be extrapolated to other farms in different parts of the world is unknown.

This study is important because it is the first to examine these associations in an area of North America where horse breeding is a prominent industry and where rhodococcal pneumonia is an ongoing cause for concern. The following findings from this study have important implications for future epidemiological studies on airborne concentrations of virulent *R equi*: (1) it appears that airborne concentrations of virulent *R equi* are not always lower in paddocks than in stalls, rather they are dependent upon whether horses are predominantly housed in the location from which the sample is collected; moreover, airborne concentrations of virulent *R equi* are significantly higher when mares and foals are present at the sampling site during sample collection; (2) the effect of a more centrally located stall versus a peripheral stall in a barn does not appear to impact on airborne concentrations of virulent *R equi*, but indicators of ventilation other than stall location need to be investigated further; (3) air samples may be collected at any time of day but the time period between 12:00 A.M. and 5:59 A.M. should be avoided because concentrations of airborne virulent *R equi* during this time are typically lower; (4) concentrations of airborne virulent *R equi* may be higher in the stalls of foals that get disease than in stalls of those that do not; (5) use of a commercially available low sodium beef broth to prepare modified NANAT agar media is an acceptable

alternative to the boiled beef broth method used by previous studies; (6) including amphotericin B in the modified NANAT agar media helps reduce fungal contaminants that are not susceptible to cycloheximide, making the morphologic identification of *R equi* colonies easier; (7) agar plates containing air samples may be stored at 4°C for up to 7 days to help reduce costs for studies that will require multiple shipments; and, (8) the packaging of agar plates for shipping from a sampling site to a laboratory should help limit the accumulation of condensation.

The extent to which the findings of this study can be extrapolated to other farms in North America is unknown. Further studies involving a larger number of farms from other regions affected by rhodococcal pneumonia are warranted to establish the relationship between airborne concentrations of virulent *R equi* and the cumulative incidence of disease and between airborne concentrations of virulent *R equi* and factors that influence those airborne concentrations. Similarly, studies involving a larger number of foals from different farms are needed to substantiate the finding that exposure to airborne virulent *R equi* during early life may be the causal link to disease development later in life.

ENDNOTES

[a] MAS-100 Eco, Merck Inc, Whitehouse Station, NJ.

[b] Skywatch ATMOS, JDC Electronics, Switzerland.

[c] Nitrocellulose membranes (pore size, 0.45 μm), Bio-Rad Laboratories, Hercules, CA.

[d] Hybridization oven, VWR International, West Chester, PA.

[e] Nonfat dry milk, Bio-Rad Laboratories, Hercules, CA.

[f] Tris-buffered saline solution, Bio-Rad Laboratories, Hercules, CA.

[g] Tween 20 solution, Bio-Rad Laboratories, Hercules, CA.

[h] Rocking platform, VWR International, West Chester, PA.

[i] Horseradish peroxidase-conjugated goat IgG fraction against mouse IgG, MP Biomedicals Inc, Aurora, OH.

[j] Sodium citrate, Sigma Chemical Co, St Louis, MO.

[k] Ethylenediaminetetraacetic acid, Sigma Chemical Co, St Louis, MO.

[l] Dextran sulfate, Sigma Chemical Co, St Louis, MO.

[m] 3,3',5,5'-tetramethylbenzidine, Sigma Chemical Co, St Louis, MO.

[n] Dulbecco phosphate buffered saline, Mediatech Inc, Manassas, VA.

[o] R, version 2.9.1, R Foundation for Statistical Computing, Vienna, Austria. Available at: cran.r-project.org/. Accessed Oct 9, 2009.

[p] Nalidixic acid sodium salt, Sigma Chemical Co, St Louis, MO.

[q] Novobiocin sodium salt minimum 90% HPLC, Sigma Chemical Co, St Louis, MO.

[r] Cycloheximide minimum 94% TLC, Sigma Chemical Co, St Louis, MO.

[s] Potassium tellurite powder, Sigma chemical Co, St Louis, MO.

[t] Swanson Beef Broth, 99% Fat Free, Campbell Soup Company, Camden, NJ.

[u] Swanson Beef Broth, 100% Fat Free, 50% Less Sodium, Campbell Soup Company, Camden, NJ.

[v] Beef Extract, MP Biomedicals Inc, Aurora, OH.

[w] Amphotericin B solubilized, Sigma Chemical Co, St Louis, MO.

[x] Brain Heart Infusion Broth, Sigma Chemical Co, St Louis, MO.

[y] SPSS 16.0 for Windows, International Business Machines, Armonk, NY.

[z] Peptone from Glycine max (soybean), Type IV, powder, Sigma Chemical Co, St Louis, MO.

[aa] Sodium chloride, ACS reagent, Sigma Chemical Co, St Louis, MO.

[bb] Yeast extract, Fluka by Sigma Chemical Co, St Louis, MO.

[cc] D-(+)- Glucose, anhydrous, Sigma Chemical Co, St Louis, MO.

[dd] Sodium dithionite, >85% (RT), Fluka by Sigma Chemical Co, St Louis, MO.

[ee] Sodium thiosulfate, 99%, Sigma Chemical Co, St Louis, MO.

[ff] Potassium phosphate dibasic powder, Sigma Chemical Co, St Louis, MO.

[gg] Sodium bicarbonate, Sigma Chemical Co, St Louis, MO.

[hh] Agar-Agar Purified, EMD Chemicals Inc., Gibbstown, NJ.

[ii] Classic collapsible 24 can cooler, Thermos L.L.C., Rolling Meadows, IL.

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APPENDIX A

Table 5. Number (percentage) of virulent *Rhodococcus equi* in air samples stratified on the basis of location on the farm from which the air sample was collected and location on the farm where mares and foals were predominantly housed for 2 horse breeding farms in central Kentucky.

		No. of virulent *R equi* (CFU/m3)			
Housing location	**Air sample location**	**0**	**1 to 3**	**4 to 6**	**>6**
Mares and foals housed predominantly in stalls at time of sample collection	Stall	141 (80)	19 (11)	12 (7)	4 (2)
	Paddock	142 (84)	23 (14)	4 (2)	0 (0)
Mares and foals housed predominantly in paddocks at time of sample collection	Stall	86 (91)	8 (8)	1 (1)	0 (0)
	Paddock	81 (90)	4 (4)	5 (6)	0 (0)

Table 6. Results of regression models for effects of location for air sample collection (paddock vs stall), location where horses were predominantly housed, time of day, humidity, ambient temperature, and wind speed on airborne concentration of virulent *R equi* at 2 horse breeding farms in central Kentucky.

Variable	Estimate*	P value†
Location of air sample, horses predominantly housed in stalls,‡ and the interaction		
Intercept	0.7	0.012
Paddock	0.5	0.133
Horses predominantly housed in stalls‡	0.5	0.088
Paddock X horses predominantly housed in stalls	-0.9	0.017
Location of air sample, horses predominantly housed in stalls, and time of day		
Intercept	0.3	0.335
Paddock	0.5	0.115
Horses predominantly housed in stalls‡	0.6	0.056
Paddock X horses predominantly housed in stalls	-1.0	0.011
6:00 A.M. to 11:59 A.M.§	0.3	0.162
Noon to 5:59 P.M.§	0.4	0.126
6:00 P.M. to 11:59 P.M.§	0.5	0.016
Location of air sample, horses predominantly housed in stalls, and time of day		
Intercept	0.5	0.382
Paddock	0.5	0.115
Horses predominantly housed in stalls‡	0.4	0.279
Paddock X horses predominantly housed in stalls	-1.0	0.027
6:00 A.M. to 11:59 A.M.§	0.4	0.091
Noon to 5:59 P.M.§	0.4	0.058
6:00 P.M. to 11:59 P.M.§	0.4	0.007
Humidity	0.001	0.820
Temperature	-0.010	0.391
Wind speed	-0.082	0.698
Wind speed X location of air sample collection	0.046	0.828

*Estimated increase in the number of CFUs/m3 for each unit change in the variable. †Values were considered significant at $P < 0.05$. ‡Reference category was horses predominantly housed in stalls. §Reference category was the period from midnight to 5:59 AM.

Table 7. Number (percentage) of air samples with positive results for virulent *R equi* as determined via modified colony immunoblots for samples collected from 3 locations at 2 horse breeding farms in central Kentucky.

Location*	Negative results	Positive results	Total
Central stalls	19 (83)	4 (17)	23 (100)
Peripheral stalls	19 (83)	4 (17)	23 (100)
Paddocks	19 (86)	3 (14)	22 (100)

*Samples were collected from 2 central stalls, 2 peripheral stalls, and 2 paddocks on each farm. A central stall was a stall most centrally located in the barn and farthest from a main entrance or any other major opening of the barn. A peripheral stall was a stall located closest to a main entrance or other major opening of the barn. A paddock was an enclosure used for housing foals.

Table 8. Results of zero-inflated Poisson mixture modeling of airborne concentration of virulent *R equi* as a function of age and *R equi* pneumonia status, obtained from stalls that housed 47 foals and their dams at a horse breeding farm in central Kentucky during 2009. 7 of 47 foals developed clinical signs of pneumonia attributed to infection with *R equi*.

Variable	Coefficient	Standard Error of Coefficient	P Value
Count data model			
Intercept	0.6898	0.2205	0.002
Age 1 week	1.4999	0.2368	<0.001
Age 2 weeks	0.9466	0.2555	<0.001
Zero values data model (model for the zero-inflation parameter)			
Intercept	0.4645	0.4001	0.2456
R equi pneumonia	1.1541	1.1742	0.3257
Age 1 week	0.4516	0.5478	0.4097
Age 2 weeks	0.4435	0.5484	0.4187
R equi pneumonia X age 1 week	-2.9870	1.4897	0.0450
R equi pneumonia X age 2 weeks	-1.1541	1.4909	0.4389

Log-likelihood = -207.3 on 9 degrees of freedom.

Table 9. Distribution (percentage) of the results of colony immunoblots for foals that did not later develop *R equi* pneumonia (no *R equi* pneumonia, N = 35) versus foals that did later develop *R equi* pneumonia (*R equi* pneumonia, N = 7) by day.

Day of Life and Disease Status	0 CFU/m3	>0 CFU/m3
Day 1 - No *R equi* pneumonia	23 (65.7)	12 (34.3)
Day 1 - *R equi* pneumonia	6 (85.7)	1 (14.3)
Day 7 - No *R equi* pneumonia	25 (71.4)	10 (28.6)
Day 7 - *R equi* pneumonia	2 (28.6)	5 (71.4)
Day 14 - No *R equi* pneumonia	25 (71.4)	10 (28.6)
Day 14 - *R equi* pneumonia	5 (71.4)	2 (28.6)

Table 10. Results of zero-inflated Poisson mixture modeling, but with disease status included in the count data model, of airborne concentration of virulent *R equi* as a function of age and *R equi* pneumonia status, obtained from stalls that housed 47 foals and their dams at a horse breeding farm in central Kentucky during 2009. 7 of 47 foals developed clinical signs of pneumonia attributed to infection with *R equi*.

Variable	Coefficient	Standard Error of Coefficient	P Value
Count data model			
Intercept	0.6996	0.2208	0.002
R equi pneumonia	-0.1386	0.1644	0.399
Age 1 week	1.5341	0.2400	<0.001
Age 2 weeks	0.9584	0.2558	<0.001
Zero values data model (model for the zero-inflation parameter)			
Intercept	0.4695	0.3989	0.2392
R equi pneumonia	1.0963	1.1854	0.3550
Age 1 week	0.4467	0.5470	0.4142
Age 2 weeks	0.4394	0.5475	0.4222
R equi pneumonia X age 1 week	-2.9298	1.4986	0.0450
R equi pneumonia X age 2 weeks	-1.1035	1.5000	0.4619

Log-likelihood = -206.9 on 10 degrees of freedom.

Table 11. Results of zero-inflated Poisson mixture modeling of airborne concentration of virulent *R equi* as a function of age and presence of mares and foals at the time of sample collection. Air samples were obtained from stalls that housed 47 foals and their dams at a horse breeding farm in central Kentucky during 2009.

Variable	Coefficient	Standard Error of Coefficient	P Value
Count data model			
Intercept	0.1660	0.2655	0.5317
Age 1 week	1.6795	0.2350	<0.001
Age 2 weeks	1.1524	0.2606	<0.001
Presence of mares and foals	0.5687	0.1698	<0.001
Zero values data model (model for the zero-inflation parameter)			
Intercept	1.0972	0.3399	0.001
Presence of mares and foals	-0.8717	0.4363	0.046

Log-likelihood = -184.0 on 6 degrees of freedom.

Table 12. Presence of the mare and/or foal in the stall at the time of day 7 sample collection and the subsequent development of *R equi* pneumonia for 35 foals.

	No *R equi* Pneumonia (N = 30)	**Developed *R equi* Pneumonia (N = 5)**
Mares/foals absent	N = 12 (40%)	N = 3 (60%)
Mares/foals present	N = 18 (60%)	N = 2 (40%)

Percentages are of column totals.

APPENDIX B

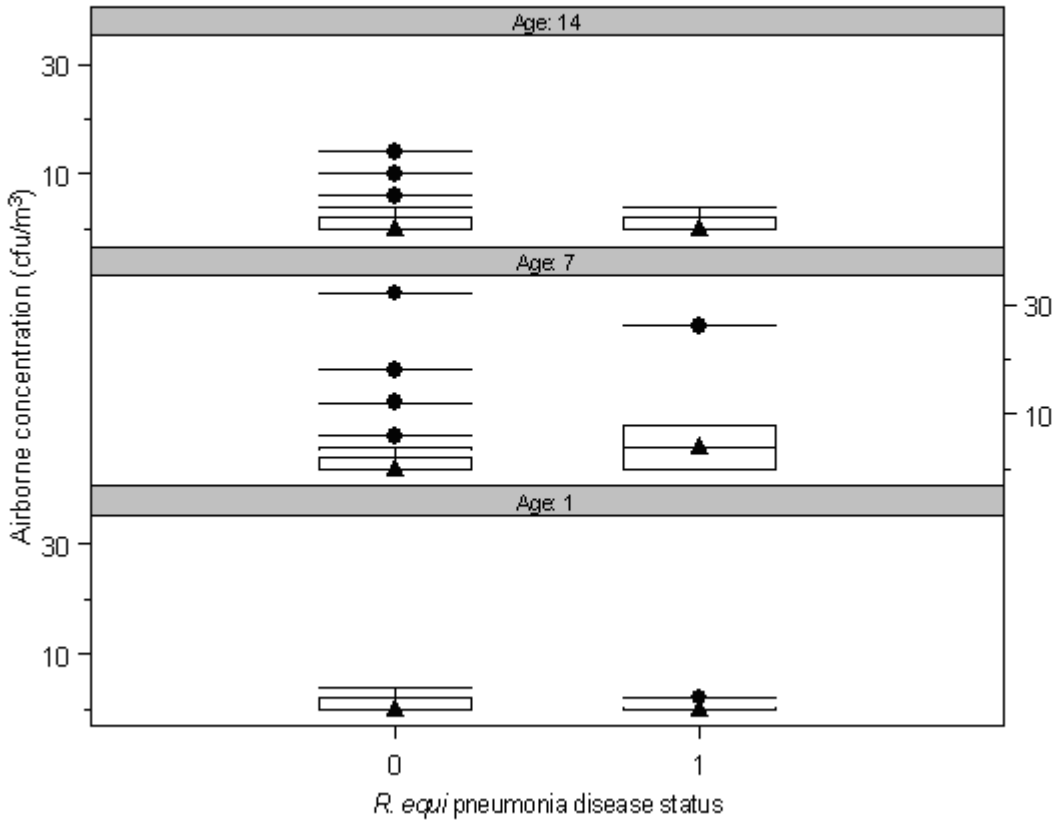

Figure 1. Box and whisker plots of the airborne concentrations of virulent *Rhodococcus equi* at age of sampling. Group 0 foals included 35 foals that did not later develop *R equi* pneumonia, whereas group 1 foals included seven foals that did later develop *R equi* pneumonia. Boxes represent the interquartile range (25th to 75th percentiles). Within each box, the horizontal line with a triangle represents the median value. Bars above the boxes extend to the 95th percentiles. The horizontal line with a circle represents an outlier.

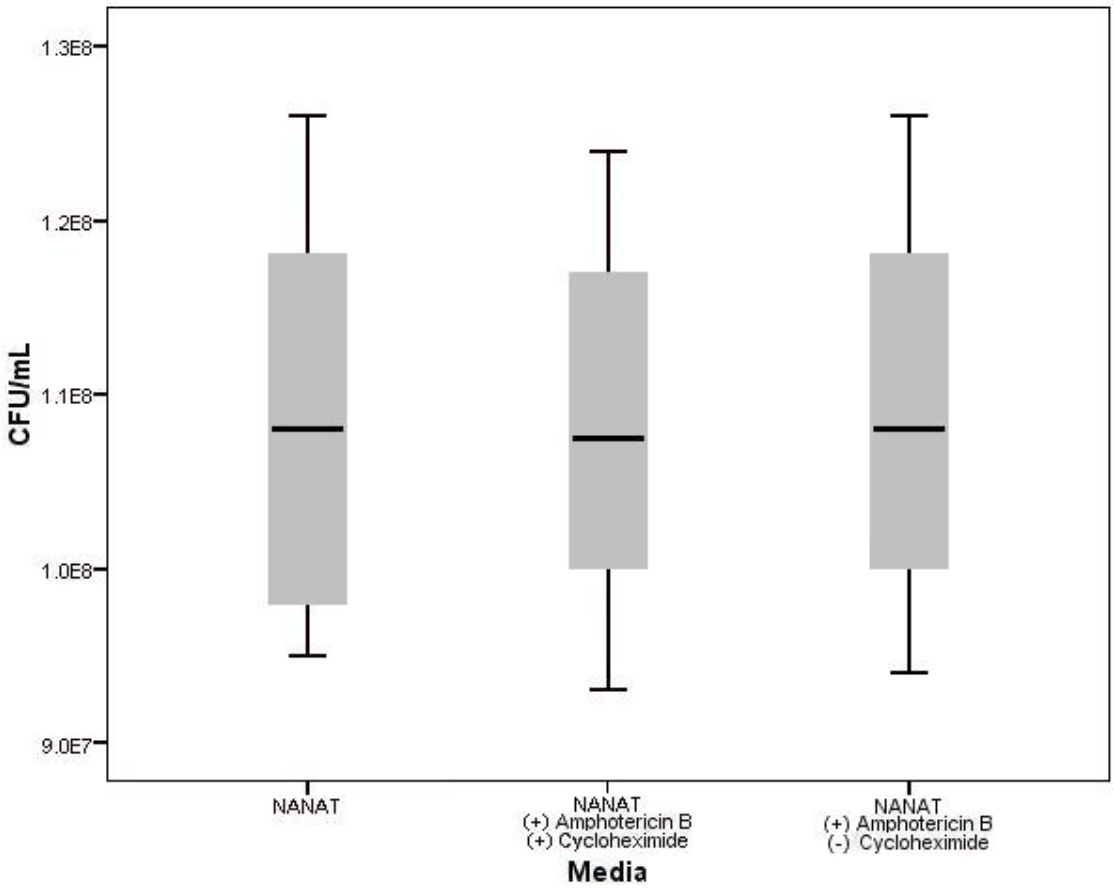

Figure 2. Box and whisker plots of the concentrations of virulent *Rhodococcus equi* (ATCC 33701) on 3 different types of modified NANAT agar media. Each group contained 18 replicates of the experiment (N = 18). Boxes represent the interquartile range (25th to 75th percentiles). Within each box, the horizontal line represents the median value. Bars above the boxes extend to the 95th percentiles.

VITA

Kyle Ryan Kuskie attended Texas A&M University from 2001 – 2007 and 2008 - 2011 and received a B.S. in Biomedical Science and an M.S. in Biomedical Sciences. During his undergraduate time, he first worked as a student worker and then as a Technician I in the Equine Infectious Disease Laboratory at Texas A&M University. In August of 2008 he entered the graduate program in the Department of Large Animal Clinical Sciences where he completed his MS in December of 2011.

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