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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

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Abstract

Objective—To determine whether airborne concentrations of virulent *R. equi* varied by housing location, time of day, and month (February through July) at 2 horse breeding farms

Sample Population—2 farms with recurrent *R. equi* foal pneumonia in central Kentucky.

Procedures—From February through July 2008, air samples were collected hourly for a 24-hour period each month from stalls and paddocks used to house mares with foals. Virulent concentrations of airborne *R. equi* were determined using a colony immunoblot technique and differences were compared using zero-inflated negative binomial methods to determine effects of location, time, and month.

Results—The presence of mares and foals at the sampling site significantly ($P = 0.017$) modified the effect of location (stall versus paddock) by increasing airborne concentrations of virulent *R. equi*. Relative to the period from midnight through 5:59 AM, airborne concentrations of virulent *R. equi* were significantly ($P = 0.016$) higher between 6:00 PM through 11:59 PM midnight. There were no significant differences in airborne concentrations between farms or among months.

Conclusions—Airborne concentrations of virulent *R. equi* were significantly increased by sampling when horses were predominately housed in the sampling site area (ie, higher in stalls when horses were predominately stalled, and higher in paddocks when horses were predominately in paddocks). For ecological and epidemiological studies, there appears to be no difference in airborne concentrations of virulent *R. equi* among air samples collected between the hours of 6:00 AM and midnight.

Keywords

Foal; *Rhodococcus equi*; epidemiology; virulence; air sampling

Rhodococcus equi, a facultative intracellular organism, is an important cause of pyogranulomatous pneumonia and lymphadenitis in foals aged 1 to 6 months, resulting in high morbidity and occasionally death.^{1,2} Virulent organisms possess an 85- to 90-kilobase plasmid that encodes the virulence-associated protein A (*vapA*) which is necessary but not sufficient to produce disease in foals.³⁻⁵

The pattern of occurrence of *R equi* pneumonia among foals varies among breeding farms from absent to sporadic to regularly recurrent (endemic).^{6,7} While previous studies have documented the presence at horse breeding farms of *R equi* in soil and feces from mares during the periparturient period, the principal source of exposure for foals remains undetermined.^{8,9} It is widely speculated that pulmonary infection results from inhalation of soil-derived virulent *R equi* found in dust from a contaminated environment.¹⁰⁻¹² Recent evidence from other continents indicates that airborne concentrations of virulent *R equi* are positively correlated with incidence of *R equi* pneumonia.^{10,13} To the authors' knowledge, studies of airborne concentrations of *R equi* have not been reported from horse farms in North America.

In this study, we analyzed the airborne concentrations of virulent *R equi* in stalls and paddocks on 2 horse breeding farms in central Kentucky. The primary aim of this study was to determine whether airborne concentrations of virulent *R equi* were associated with any combination of the following factors: 1) farm location (stall versus paddock); 2) presence of horses at the location at the time of sampling; 3) time of day; 4) month of year; 5) ambient temperature; 6) relative humidity; and, 7) wind speed. As a secondary aim, we investigated whether a more central (ie, stalls most remote from a main opening of the barn) or peripheral (ie, stalls closest to a main opening) location in the barn impacted airborne concentrations of *R equi* in stalls.

Materials and Methods

Farms and sampling location

Two Thoroughbred horse breeding farms in central Kentucky with a history of recurrent cases of *R equi* foal pneumonia (ie, *R. equi*-endemic farms) were used in the study. The rationale for sampling air at 2 endemic farms was on the basis of the assumption that airborne concentrations are positively associated with incidence of disease, such that using *R. equi*-endemic farms would provide relatively fewer samples yielding negative results (no growth of *R equi*).¹⁰

A single centrally-located stall in a barn housing newborn foals, and a single outdoor paddock used to maintain mares and foals housed in that barn, were sampled monthly from February 2008 through July 2008 at each farm. Hourly samples for a 24-hour period were collected from each location at each farm during the first week of the month. Paddock samples were collected within 10 yards of the gate, as 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 monthly samples were collected at a single time-point (3:00 PM) from 2 peripheral stalls, 2 central stalls, and 2 paddocks used for housing foals at each farm. Peripheral stalls were selected as those closest to a main entrance to the barn and central stalls were selected as those furthest from a main entrance or any other major opening of the barn. The sampling location in the additional paddocks was the same as previously described.

Air sample collection

A portable, commercially available, air sampling device^a was used for collecting air samples. Modified NANAT culture plates (100-mm), a selective media commonly used for epidemiological studies of *R equi*,^{9,14} were used during sampling. The air sampler was placed on the ground to collect air at a height of approximately 10 centimeters above the stall floor or paddock ground. For each sample collection, 500 liters of air was aspirated onto a modified NANAT culture plate, at a rate of 100 liters per minute. Before each sample was collected, the sieve of the air sampler was disinfected using an isopropanol wipe.¹³ Ambient temperature, relative humidity, and windspeed were also recorded at the time of each individual sampling using another handheld device.^b At all sampling times, horses were predominately maintained either in stalls in the barn or in paddocks. When each sample was collected, it was recorded whether mares and foals were predominately (ie, > 80% of the mare and foal population for the barn being sampled) housed in stalls or barns at the time of sample collection; however, presence at the time of sampling of a mare or foal in a particular stall that was sampled was not recorded.

Modified colony immunoblot assay

Culture plates were transported chilled with icepacks in insulated containers to the Equine Infectious Disease Laboratory (EIDL) at Texas A&M University. Upon arrival, plates were incubated at 37 °C for 48 hours. Following incubation, plates were analyzed for the presence of *R equi*, based on morphological characteristics. The plates that were determined to have *R equi* present were then tested for virulence.

The concentration of virulent *R equi* in each air sample was determined by using a modified immunoblotting technique for detection of VapA.^{9,15} This method allows for the quantification of virulent *R equi* within a background of bacterial and fungal contamination. Nitrocellulose membranes^c were placed onto previously incubated modified NANAT culture plates, containing at least 1 colony forming unit (CFU) of *R equi*, and allowed to become completely saturated. Once saturated, membranes were removed and air-dried for 30 min at room temperature and then baked in a hybridization oven^d at 100°C for 1 min. Membranes were then 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 in TBSS with 0.05% Tween 20^g at 10 min per wash. Membranes were incubated overnight at 4°C on a rocking platform^h with the monoclonal antibody (provided by 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 IgGⁱ, 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 min at 37°C, and 3 washes (5 min/wash) with citrate-EDTA buffer at 37°C. The

^aMAS-100 Eco™, Merck, Inc., Whitehouse Station, NJ

^bSkywatch ATMOS, JDC Electronics, Switzerland

^cNitrocellulose membranes, pore size 0.45 µm, Bio-Rad Laboratories, Hercules, Calif.

^dHybridization oven, VWR International, West Chester, Pa.

^eNonfat dry milk, Bio-Rad Laboratories, Hercules, Calif.

^fTris-buffered saline solution, Bio-Rad Laboratories, Hercules, Calif.

^gTween 20 solution, Bio-Rad Laboratories, Hercules, Calif.

^hRocking platform, VWR International, West Chester, Pa.

ⁱHorseradish peroxidase-conjugated goat IgG fraction against mouse IgG, MP Biomedicals Inc, Aurora, Ohio.

^jSodium citrate, Sigma Chemical Co, St. Louis, Mo.

^kEthylenediaminetetraacetic acid, Sigma Chemical Co, St. Louis, Mo.

^lDextran sulfate, Sigma Chemical Co, St. Louis Mo.

substrates 3,3',5,5'-tetramethylbenzidine^m and hydrogen peroxide were added to the membranes and then incubated at 37°C for 30 min, or until development of the positive control membrane was sufficient. 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 expressed as CFU per cubic meter of air (CFU/m³), calculated by the following equation:

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

In the equation, *C* is the airborne concentration of virulent *R equi*, *T* is the total of virulent *R equi* colonies counted on the membrane, *t* is the total sampling time, and *F* is the rate of airflow of the sampling device¹⁶.

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 media¹⁷ and ten-fold serial dilutions were performed by using phosphate buffered saline solutionⁿ. One hundred µL of the dilution containing approximately 10⁴ CFUs of *R equi*/mL was plated for each strain. Colony immunoblot control plates were incubated under the same conditions as the airborne sample plates.

Data analysis

The airborne concentration of virulent *R equi*, denoted as *C*, were counts and consisted of values of 0 and non-0, positive integers. Because most air samples yielded no isolates of virulent *R equi*, the data set contained a large proportion of 0s. Consequently, we used a zero-inflated negative binomial (ZINB) model to analyze the airborne concentration *C*. The ZINB model is a mixture of 2 components: a negative binomial (NB) distribution for the unbounded counts, and a point-mass at zero to account for the inflated number of zeros. The NB component is chosen due to its enhanced flexibility relative to the commonly used zero-inflated Poisson model¹⁸ 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 non-0 counts than the Poisson model. We model the 0 versus 1 mixture by a logistic regression model while we let the mean of NB distribution be a linear combination of the variables under investigation. The analyses were performed using the EM algorithm for mixture modeling, as described as a general methodology in McLachlan and Peel.¹⁹ All coding was done using the R^o computing platform. A value of *P* < 0.05 was used for significance.

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 using chi-squared or Fisher's exact tests with value of *P* < 0.05 considered as significant. The rationale for using the dichotomous outcome was that most air samples yielded no isolates of virulent *R equi* (i.e., data had many 0s) and because the statistical power for ZINB modeling was greatly restricted by the small sample size for these comparisons.

^m3,3',5,5'-tetramethylbenzidine, Sigma Chemical Co, St. Louis, Mo.

ⁿDulbecco's Phosphate Buffered Saline, Mediatech, Inc, Manassas, VA

^oR, <http://cran.r-project.org/>

Results

A total of 557 samples of 576 (96%) possible samples were collected to evaluate effects of farm location, presence of horses at the sampled location, time of day, humidity, temperature, and windspeed. Twenty-nine (5%) samples were unavailable for the following reasons: 10 samples weren't collected because of battery failure of the air sampler and 19 samples were damaged during shipping to the EIDL. 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 analysis.

Location effects

The effects of farm location (paddock versus stall) were significantly modified by whether or not horses were being housed primarily in the barn at the time of sampling. Although concentrations tended to be higher in paddocks than in stalls, the difference was not significant (Table 1.a.). The effect of horses being stalled when samples were collected significantly ($P = 0.017$) modified airborne concentrations of virulent *R equi*: when horses were predominately housed in stalls in the barn, airborne concentrations of virulent *R equi* were higher in samples collected from stalls, and when horses were predominately housed in paddocks, airborne concentrations of virulent *R. equi* were higher in samples collected from paddocks. The proportion of stalls yielding concentrations of ≥ 4 CFU/m³ was approximately 9% when horses were primarily stalled compared to 1% when horses were not stalled; in contrast, the proportion of paddocks yielding concentrations of ≥ 4 CFU/m³ was approximately 2% when horses were primarily stalled compared to approximately 5% when horses were not stalled. There were no significant differences between farms in magnitude or significance effects of farm location (stall versus paddock) or where horses were predominately maintained (stalled versus in paddocks) on airborne concentrations of virulent *R equi*.

Effects of time of day

On the basis of exploratory data analysis, time of day was categorized into 4 periods: 1) 12:00 AM to 5:59 AM; 2) 6:00 AM to 11:59 AM; 3) 12:00 PM to 5:59 PM; and, 4) 6:00 PM to 11:59 PM. Relative to the period from 12:00 AM to 5:59 AM, concentrations tended to increase for later periods and were significantly ($P = 0.016$) greater for the period from 6:00 PM through 11:59 PM (Table 1.b.), using multivariate methods to adjust for the effects of farm location and where horses were primarily housed, and their interaction.

Effect of month of year

Samples were collected monthly between February and July, 2008. There was no significant effect of month on airborne concentrations, after adjusting for effects of farm location, whether horses were primarily stalled, and their interaction.

Effects of windspeed, humidity, and ambient temperature

Windspeed, humidity, and ambient temperature were recorded at the time each air sample was collected. There were no significant effects of windspeed, humidity, or ambient temperature on the airborne concentrations after accounting for effects of farm location, whether horses were primarily stalled, and their interaction (Table 1.c.) A term for windspeed by farm location was forced into the model a priori because of our belief that windspeed 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 significance and magnitude of effects when the interaction term was not included and whether windspeed, humidity, and temperature were considered individually as covariates.

Comparison of central versus peripheral stalls and paddocks

Additional samples were collected monthly at a single time-point (3:00 PM) 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 samples, 68 (94%) were collected from the 2 farms and available for testing. Of these 68 samples, 11 (16%) yielded positive results for virulent *R equi*. The frequency of air samples positive for virulent *R equi* did not differ significantly by sampling site (Table 2). The distribution did not differ significantly between farms: the numbers of positive samples from central stalls, peripheral stalls, and paddocks at farm 1 were 2, 3, and 1, respectively, and at farm 2 were 2, 1, and 2 respectively. 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 CFU/m³ in central stalls, 16 CFU/m³ in peripheral stalls, and 8 CFU/m³ in paddock samples.

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 evaluating airborne concentration of virulent *R equi* can advance our understanding of the epidemiology of *R equi* pneumonia at horse breeding farms.^{10,12} Following on the seminal work evaluating airborne concentrations of *R equi* at horse farms in Australia and Ireland,^{10,12} the purpose of the study reported here was to provide data regarding the effects of time of day, farm location, and environmental/climatic conditions on airborne concentrations of virulent *R equi* at horse breeding farms in North America.

A previous study indicated that airborne concentrations of virulent *R equi* were higher in stalls than on paddocks at 3 Irish breeding farms.¹⁰ In the study reported here, the association of airborne concentration of virulent *R equi* with housing location (stall versus paddock) was dependent upon where horses were being predominately housed. When horses were being housed primarily in stalls in the barn, airborne concentrations of virulent *R equi* were significantly lower in paddocks than stalls; conversely, when horses were not being housed primarily in the barn, airborne concentrations were higher in the paddock. The reason for this effect modification (ie, significant interaction of farm location and where horses primarily were being housed) was not determined, but it might be explained by the physical activity of mares and foals near the sampling site causing more matter from the environment, including virulent *R equi*, to become aerosolized/airborne. Further studies are needed to test the hypotheses that increased density of horses or level of activity that disrupts the ground surface results in increased airborne concentrations of *R equi*. Conceivably, results of these studies might partly explain the observed positive association between cumulative incidence of *R equi* foal pneumonia and the density of horses or foals at breeding farms.^{8,20} An important limitation of the study reported here was that we failed to record whether mares and foals were present in the stall from which air was being collected at the time of sampling.

There were no significant differences between farms in the association of concentration of airborne *R equi* and farm location, whether horses were stalled, or their interaction. This was not surprising because the study's sample population was 2 farms that experienced recurrent cases of foals affected with *R equi* pneumonia. These farms were similar in regard to 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 this study are relevant to farms that differ in acreage, number of horses, management practices, and geographical location.

The time of day appeared to influence the airborne concentration of virulent *R equi*. Using 12:00 AM to 5:59 AM as the reference period, airborne concentrations of virulent *R equi* tended to increase during the day, being significantly higher ($P=0.016$) during the time period from 6:00 PM to 11:59 PM; there were no significant differences in airborne concentrations of virulent *R equi* among later 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, moving horses to and from paddocks, etc.) and horses in the areas sampled during the 3 later periods of the day. From the standpoint of epidemiological studies, it appears that sampling between 6 AM and midnight is preferable for recovering *R equi*, and that marked variation in results collected during regular working hours would be unlikely. From the standpoint of disease control, it might be possible to reduce exposure of virulent *R equi* to foals by reducing activities of people (eg, sweeping aisles, cleaning stalls) in barns (or paddocks) when foals are in these areas.

There were no significant effects of sampling month on airborne concentrations, either when considering month of the year as the sole parameter used for modeling, or when adjusting for farm location, horses being housed primarily in the barn, and time of day. In Australia, airborne concentrations of virulent *R equi* were higher during the later (and warmer) months of their 6-month study during foaling season.¹² The reasons for this discrepancy between the 2 studies are unclear, but might be related to the relatively modest magnitude of the observed effects from Australia (estimated odds ratios of about 2, with lower bounds of the 95% confidence intervals of those odds ratios of about 1.2 and 1.4), and the considerably smaller scope of our study (involving only 2 farms, rather than the 22 farms studied in Australia) which limited statistical power. Further evaluation of the effect of month on the airborne concentration of virulent *R equi* is needed.

During March 2008, 1 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 the 24 (4%) air samples collected from stalls at this farm yielded virulent *R equi*, and the concentration for the positive sample was 2 CFU/m³. Moreover, the numbers of non-rhodococcal bacteria growing on these 24 plates appeared to be much less than those seen during any other months. This finding suggests that farms could significantly decrease airborne concentrations of bacteria, and possibly the occurrence of multiple airborne diseases, by routinely disinfecting barns. The use of decontamination of bedding or the barn/stable to reduce the incidence or severity of *R equi* pneumonia has been proposed previously.¹⁰

Results showed no significant effects of windspeed, humidity, or temperature on the airborne concentration of virulent *R equi*. This finding also was in contrast to those of previous studies.^{10,11} Takai *et al.*¹¹ reported that the number of *R equi* isolated from the air increased on dry and windy days. However, the sampling technique used for that study was markedly different than that used for this study, and this methodological discrepancy could contribute to the conflicting results. In addition, that study examined the concentration of all environmental *R equi*, rather than examining only concentrations of virulent organisms.¹¹ It is also possible that the range of windspeed, humidity, and temperature was more variable for other studies, or that our sample size was too small to detect differences that were apparent when studying a larger number of farms in different regions of Australia.¹⁰ Further evaluation of the effects of humidity, temperature, and windspeed at a larger number of farms in North America is warranted.

As a secondary aim, we hypothesized that, because of differences in ventilation, stalls located more remotely from doorways/major openings to the barn might have higher

airborne concentrations of virulent *R equi* than stalls located adjacent to openings. However, when samples collected at approximately the same time of day (ie, within 1 hour) were collected from 2 central stalls, 2 peripheral stalls, and 2 paddocks, there were no significant differences in the frequency of positive samples by 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 using a larger sample size and more accurate methods for assessing the ventilation of the area sampled.

In summary, it appears that airborne concentrations of virulent *R equi* are associated with farm location (stall versus paddock) in a manner that depends on whether horses are predominately housed in the location from which the sample is collected, and that airborne concentrations are lowest during the period between 12:00 AM and 5:59 AM. Further studies in North America to establish the relationship between airborne concentrations of virulent *R equi* and both the cumulative incidence of disease and factors that influence the airborne concentration of *R equi* are warranted, including the influence of the presence and activity level of horses and humans and the density of horses (and foals) per acre. The results of this study indicate that these associations may be complex, and that sampling should best be performed after 6:00 AM and before midnight.

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Table 1

Results of regression models for effects on airborne concentration of virulent *R equi* of farm location (paddock versus stall [reference category]), whether horses were primarily being housed in stalls, time of day, windspeed, humidity, and ambient temperature

<i>a. Effects of location, horses primarily stalled, and their interaction</i>		
Variable	Estimate *	P value
Intercept	0.7	0.012
Paddock	0.5	0.133
Horses primarily stalled	0.5	0.088
Paddock x horses primarily stalled (interaction term)	-0.9	0.017
<i>b. Effects of location, horses primarily stalled, and daily time-period</i>		
Variable	Estimate *	P value
Intercept	0.3	0.335
Paddock	0.5	0.115
Horses predominately stalled	0.6	0.056
Paddock x horses predominantly stalled (interaction term)	-1.0	0.011
6 AM to 11 AM **	0.3	0.162
12 PM to 5 PM **	0.4	0.126
6 PM to 11 PM **	0.5	0.016
<i>c. Effects of location, horses primarily stalled, and daily time-period</i>		
Variable	Estimate *	P value
Intercept	0.5	0.382
Paddock	0.5	0.115
Horses predominately stalled	0.4	0.279
Paddock x horses predominantly stalled (interaction term)	-1.0	0.027
6 AM to 11 AM **	0.4	0.091
12 PM to 5 PM **	0.4	0.058
6 PM to 11 PM **	0.5	0.007
Humidity	0.001	0.820
Temperature	-0.010	0.391
Windspeed	-0.082	0.698
Windspeed by location	0.046	0.828

* Estimated increase in CFU/m³ per unit change in variable

** Reference category was the period from 12 AM through 5 AM

Table 2

Frequency distribution of air samples positive for virulent *R equi* by colony immunoblot collected from 3 sites (peripheral stall, central stall, or paddock [see text for details]) at 2 farms in central Kentucky

Location	No. negative (%)	No. positive (%)	Total
Central stall	19 (83%)	4 (17%)	23
Peripheral stall	19 (83%)	4 (17%)	23
Paddock	19 (86%)	3 (14%)	22