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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Objective—To determine whether airborne concentrations of virulent *Rhodococcus equi* at 2 horse breeding farms varied on the basis of location, time of day, and month.

Sample Population—2 farms in central Kentucky with recurrent *R equi*–induced pneumonia in foals.

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 and their foals. Concentrations of airborne virulent *R equi* were determined via a modified colony immunoblot technique. Differences were compared by use of zero-inflated negative binomial methods to determine effects of location, time, and month.

Results—Whether mares and foals were housed predominantly in stalls or paddocks significantly affected results for location of sample collection (stall vs paddock) by increasing airborne concentrations of virulent *R equi* at the site where horses were predominantly housed. Airborne concentrations of virulent *R equi* were significantly higher from 6:00 PM through 11:59 PM than for the period from midnight through 5:59 AM. Airborne concentrations of virulent *R equi* did not differ significantly between farms or among months.

Conclusions and Clinical Relevance—Airborne concentrations of virulent *R equi* were significantly increased when horses were predominantly housed at the site for collection of air samples (ie, higher in stalls when horses were predominantly housed in stalls and higher in paddocks when horses were predominantly housed in paddocks). Concentrations of virulent *R equi* among air samples collected between the hours of 6:00 AM and midnight appeared similar. (*Am J Vet Med* 2011;72:73–79)

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.[1,2] Virulent organisms possess an 85- to 90-kilobase plasmid that encodes virulence-associated protein A, which is necessary but not sufficient to cause disease in foals.[3–5]

The pattern of *R equi*–induced pneumonia among foals varies among breeding farms from absent to sporadic to regularly recurrent (endemic).[6,7] Although investigators in other studies[8,9] 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 environment.[10–12] Evidence from studies[10,13] conducted on other continents indicates that airborne

Abbreviations

NB	Negative binomial
TBSS	Tris-buffered saline solution
ZINB	Zero-inflated negative binomial

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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*).[10]

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 PM) 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*,[9,14] 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.[13] 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.[9,15] This method allowed for the quantification of virulent *R equi* within a background of bacterial and fungal contamination. Nitrocellulose membranes[c] containing at least 1 CFU of *R equi* were placed onto previously incubated culture plates that contained 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.[f] Membranes were incubated overnight at 4°C on a rocking platform[g] 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[h] (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[i] and 10mM EDTA[i] [pH, 5.0]) for 5 minutes at 37°C, 1 wash with citrate-EDTA buffer and 1% dextran sulfate[i] 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[i] 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[16]:

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

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,[17] and 10-fold serial dilutions were performed by use of PBS solution.[j] One hundred microliters of the dilution containing approximately 10^4 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 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 NB component was chosen because of its enhanced flexibility relative to the commonly used zero-inflated Poisson model[18] 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.[19] All coding was performed by use of a computing platform.[k] 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 1**). Concentrations typically were higher in paddocks than in stalls, although the difference was not significant (**Table 2**). 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%)

Table 1—Number (percentage) of virulent *Rhodoccus 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* (CFUs/m^3)			
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	143 (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 2—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 × 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 × horses predominantly housed in stalls	–1.0	0.011
6:00 AM to 11:59 AM§	0.3	0.162
Noon to 5:59 PM§	0.4	0.126
6:00 PM to 11:59 PM§	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 × horses predominantly housed in stalls	–1.0	0.027
6:00 AM to 11:59 AM§	0.4	0.091
Noon to 5:59 PM§	0.4	0.058
6:00 PM to 11:59 PM§	0.5	0.007
Humidity	0.001	0.820
Temperature	–0.010	0.391
Wind speed	–0.082	0.698
Wind speed × location of air sample collection	0.046	0.828

*Estimated increase in the number of CFUs/m^3 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.

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 AM, 6:00 AM to 11:59 AM, noon to 5:59 PM, and 6:00 PM to 11:59 PM). Compared with concentrations of virulent *R equi* for the period from midnight to 5:59 AM, concentrations typically were higher at other time periods during the day and were significantly ($P = 0.016$) higher for the period from 6:00 PM through 11:59 PM (Table 2), 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 houses were predominantly housed, and the interaction of these 2 terms (Table 2). 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.

Table 3—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.

Central stalls, 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 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 3**). 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[10,12] 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 climatic 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.[10] 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.[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 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 hectares, 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 AM 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 PM to 11:59 PM 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 AM 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 of 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[12] 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 the positive results was 2 CFUs/m^3. This distribution of samples with positive results appeared markedly lower than the cumulative distribution for stalls (Table 1). 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 diseases, 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.[10]

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.[10,11] Authors in one of those studies[11] reported that the number of *R equi* isolated from the air increased on dry and windy days. However, the sample collection 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[11] 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 the 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 AM. 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 AM and midnight.

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, Calif.
d. Hybridization oven, VWR International, West Chester, Pa.
e. Nonfat dry milk, Bio-Rad Laboratories, Hercules, Calif.
f. Bio-Rad Laboratories, Hercules, Calif.
g. Rocking platform, VWR International, West Chester, Pa.
h. Horseradish peroxidase–conjugated goat IgG fraction against mouse IgG, MP Biomedicals Inc, Aurora, Ohio.
i. Sigma Chemical Co, St Louis, Mo.
j. Dulbecco phosphate buffered saline, Mediatech Inc, Manassas, Va.
k. R, version 2.9.1, R Foundation for Statistical Computing, Vienna, Austria. Available at: cran.r-project.org/. Accessed Oct 9, 2009.

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